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(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK–2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BJØRNVAD, Mads [DK/DK]; Novo Nordisk a/s, Novo Allé, DK–2880 Bagsværd (DK). PEDERSEN, Sven [DK/DK]; Novo Nordisk a/s, Novo Allé, DK–2880 Bagsværd (DK). SCHULEIN, Martin [DK/DK]; Novo Nordisk a/s, Novo Allé, DK–2880 Bagsværd (DK). BISGÅRD–FRANTZEN, Henrik [DK/DK]; Novo Nordisk a/s, Novo Allé, DK–2880 Bagsværd (DK).

(74) Common Representative: NOVO NORDISK A/S; Novo Allé, DK–2880 Bagsværd (DK).

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(54) Title: ALPHA–AMYLASE FUSED TO CELLULOSE BINDING DOMAIN, FOR STARCH DEGRADATION

(57) Abstract

The invention relates to a starch conversion method wherein the starch substrate is treated in aqueous medium with an CBD/enzyme hybrid. Further, the invention also relates to an isolated DNA sequence encoding a stable CBD/enzyme hybrid, a DNA construct comprising said DNA sequence of the invention, an expression vector comprising the DNA sequence of the invention, and a CBD/enzyme hybrid.

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ALPHA-AMYLASE FUSED TO CELLULOSE BINDING DOMAIN, FOR STARCH DEGRADATION

FIELD OF THE INVENTION

The present invention relates, *inter alia*, to the use of a hybrid between a carbohydrate-binding domain ("CBD") and an enzyme of a type employed in industrial starch processing [notably starch processing for the production (*vide infra*) of sweeteners, particularly glucose- and/or fructose-containing syrups], especially an amylolytic enzyme, such as an α-amylase employed in a so-called "starch liquefaction" process (*vide infra*) in which starch is degraded (often termed "dextrinized") to smaller oligo- and/or polysaccharide fragments, or a debranching enzyme (such as an isoamylase or a pullulanase) employed to debranch amylopectin-derived starch fragments in connection with the so-called "saccharification" process (*vide infra*) which is normally carried out after the liquefaction stage. The invention also relates to hybrid enzyme consisting of a CBD-linker-enzyme.

BACKGROUND OF THE INVENTION

As indicated above, the present invention is of particular value in the field of starch processing (starch conversion). Conditions for conventional starch conversion processes and for liquefaction and/or saccharification processes are described in, e.g., US 3,912,590 and in EP 0,252,730 and EP 0,063,909.

Production of sweeteners from starch:

A "traditional" process for the production of glucose- and fructose-containing syrups from starch normally consists of three consecutive enzymatic processes, viz. a liquefaction process followed by a saccharification process and (for production of fructose-containing syrups) an isomerization process. During the liquefaction process, starch (initially in the form starch suspension in aqueous medium) is degraded to dextrins (oligo- and polysaccharide fragments of starch) by an α-amylase [EC 3.2.1.1; e.g. Termamyl™ (*Bacillus licheniformis* α-amylase), available from Novo Nordisk A/S, Bagsvaerd, Denmark], typically at pH

values between 5.5 and 6.2 and at temperatures of 95-160°C for a period of approximately 2 hours. In order to ensure optimal enzyme stability under these conditions, approximately 1mM of calcium (ca. 40 ppm free calcium ions) is typically added to the starch suspension.

After the liquefaction process the dextrins are converted into dextrose (D-glucose) by addition of a glucoamylase (amyloglucosidase, EC 3.2.1.3; e.g. AMG™, from Novo Nordisk A/S) and, typically, a debranching enzyme, such as an isoamylase (EC 3.2.1.68) or a pullulanase (EC 3.2.1.41; e.g. Promozyme™, from Novo Nordisk A/S). Before this step the pH of the medium is normally reduced to a value below 4.5 (e.g pH 4.3), maintaining the high temperature (above 95°C), and the liquefying α-amylase activity is thereby denatured. The temperature is then normally lowered to 60°C, and glucoamylase and debranching enzyme are added. The saccharification process is normally allowed to proceed for 24-72 hours.

After completion of the saccharification stage, the pH of the medium is increased to a value in the range of 6-8, preferably pH 7.5, and calcium ions are removed by ion exchange. The resulting syrup (dextrose syrup) may then be converted into high fructose syrup using, e.g., an immobilized "glucose isomerase" (xylose isomerase, EC 5.3.1.5; e.g. Sweetzyme™, from Novo Nordisk A/S).

A number of improvements in the properties of enzymes currently employed in starch conversion processes would be desirable. With respect to starch liquefaction, employing liquefying α-amylases, at least 3 improvements could be envisaged and are outlined below; each of these could be regarded as an individual benefit, although any combination (e.g. 1+2, 1+3, 2+3 or 1+2+3) could advantageously be employed:

Improvement 1.

Reduction of the Ca^{2+} dependency of the liquefying α-amylase.

Addition of free calcium (calcium ion) is required to ensure adequately high stability of α-amylases currently employed for starch liquefaction, but the presence of calcium ions in the

medium at the isomerization stage results in strong inhibition of the activity of the glucoseisomerase employed therein. It is therefore necessary either to reduce the calcium ion content of the medium, by means of an expensive unit operation (e.g. ion exchange), to a level below about 3-5 ppm of free calcium, or to minimize the inhibitory effect of calcium in some other manner, e.g. by addition, after the saccharification stage, to the medium of magnesium ions in a amount sufficient to adequately "out-compete" binding of calcium to the glucoseisomerase. Significant savings could be achieved if the liquefaction process could be performed without addition of calcium ions, thereby eliminating the need for subsequent, expensive remedial unit operations to remove calcium or minimize the inhibitory effect thereof.

To achieve this, an α-amylolytic enzyme which is stable and highly active at low concentrations of free calcium (< 40 ppm) is required. Such an enzyme should preferably have a pH optimum at a pH in the range of 4.5-6.5, more preferably in the range of 4.5-5.5.

Improvement 2.

Reduction of formation of unwanted Maillard products.

The extent of formation of unwanted Maillard products during the liquefaction process is dependent on the pH. Low pH favours reduced formation of Maillard products. It would thus be desirable to be able to lower the process pH from around pH 6.0 to a value around pH 4.5; unfortunately, all commonly known, thermostable liquefying α-amylases are not very stable at low pH (i.e. pH < 6.0) and their specific activity is generally low.

Achievement of the above-mentioned goal requires the availability of an α-amylolytic enzyme which is stable at a pH in the range of 4.5-5.5, and which preferably maintains a high specific activity.

Improvement 3.

Reduced influence of the liquefying α-amylase on the saccharification process.

It has been reported previously (US patent 5,234,823) that

when saccharifying with *A. niger* glucoamylase and *B. acidopullulyticus* pullulanase, the presence of residual α-amylase activity remaining after the liquefaction process can lead to lower yields of dextrose if the α-amylase is not inactivated before the saccharification stage. As already mentioned (*vide supra*), this inactivation is typically carried out by adjusting the pH to below 4.5 at 95°C, before lowering the temperature to 60°C for saccharification.

The cause of this negative effect on dextrose yield is not fully understood, but it is assumed that the liquefying α-amylase preparation employed (e.g. a Termamyl™ product, such as Termamyl™ 120 L) generates "limit dextrins" (which are poor substrates for *B. acidopullulyticus* pullulanase) by hydrolysing 1,4-alpha-glucosidic linkages close to and on both sides of the branching points in amylopectin. Hydrolysis of these limit dextrins by glucoamylase leads to a build-up of the trisaccharide panose, which is only slowly hydrolysed by glucoamylase.

The development of a thermostable α-amylolytic enzyme which does not suffer from this disadvantage would be a significant process improvement, as no separate inactivation step would be required.

One object of the present invention is to achieve improved performance of α-amylolytic enzymes in relation to starch liquefaction processes - e.g. by achieving one or more or the above-outlined improvements - by changing the affinity of the enzyme for the starch substrate, whereby the modified enzyme comes into more intimate contact with the substrate.

SUMMARY OF THE INVENTION

One aspect of the invention relates to an improved enzymatic process for liquefying starch employing a modified form of a liquefying α-amylase, wherein the α-amylase in question is linked to an amino acid sequence comprising a carbohydrate-binding domain (*vide infra*).

The invention also relates to an improved enzymatic process for liquefying starch which besides a modified α-amylase also is

treated with a debranching enzyme. The debranching enzyme may be modified by linkage to an amino acid sequence comprising a carbohydrate-binding domain.

Similarly, and also within the scope of the invention, it is envisaged that the use of an analogously modified (i.e. CBD-derivatized) form of a debranching enzyme, such as an isoamylase or a pullulanase, for debranching amylopectin-derived starch fragments (e.g. in connection with the above-outlined saccharification stage of a starch conversion process) will result in enhanced debranching performance, and thereby dextrose yield improvement, in the saccharification procedure.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect the present invention thus relates to a method for liquefying starch, wherein a starch substrate is treated in aqueous medium with a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of an α-amylase linked (i.e. covalently bound) to an amino acid sequence comprising a carbohydrate-binding domain (CBD).

The invention also relates to an improved enzymatic process for liquefying starch which besides a modified α-amylase also is treated with a debranching enzyme. The debranching enzyme may be modified by linkage to an amino acid sequence comprising a carbohydrate-binding domain.

A further aspect of the present invention relates to a method for saccharifying starch which has been subjected to a liquefaction process, wherein the reaction mixture after liquefaction is treated with a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of an amylopectin-debranching enzyme (e.g. an isoamylase or a pullulanase) linked (i.e. covalently bound) to an amino acid sequence comprising a carbohydrate-binding domain (CBD).

It is to understood that starch liquefaction processes as referred to in the context of the present invention do not embrace, for example, textile de-sizing processes wherein starch ("size") present in fabrics or textiles (normally cellulosic or cellulose-containing fabrics or textiles) is removed from the

fabric or textile by an enzymatic process.

Carbohydrate-binding domains

A carbohydrate-binding domain (CBD) is a polypeptide amino acid sequence which binds preferentially to a poly- or oligosaccharide (carbohydrate), frequently - but not necessarily exclusively - to a water-insoluble (including crystalline) form thereof.

Although a number of types of CBDs have been described in the patent and scientific literature, the majority thereof - many of which derive from cellulolytic enzymes (cellulases) - are commonly referred to as "cellulose-binding domains"; a typical cellulose-binding domain will thus be a CBD which occurs in a cellulase. Likewise, other sub-classes of CBDs would embrace, e.g., chitin-binding domains (CBDs which typically occur in chitinases), xylan-binding domains (CBDs which typically occur in xylanases), mannan-binding domains (CBDs which typically occur in mannanases), starch-binding domains [CBDs which may occur in certain amylolytic enzymes, such as certain glucoamylases, or in enzymes such as cyclodextrin glucanotransferases ("CGTases")], and others.

CBDs are found as integral parts of large polypeptides or proteins consisting of two or more polypeptide amino acid sequence regions, especially in hydrolytic enzymes (hydrolases) which typically comprise a catalytic domain containing the active site for substrate hydrolysis and a carbohydrate-binding domain (CBD) for binding to the carbohydrate substrate in question. Such enzymes can comprise more than one catalytic domain and one, two or three CBDs, and optionally further comprise one or more polypeptide amino acid sequence regions linking the CBD(s) with the catalytic domain(s), a region of the latter type usually being denoted a "linker". Examples of hydrolytic enzymes comprising a CBD - some of which have already been mentioned above - are cellulases, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. CBDs have also been found in algae, e.g. in the red alga *Porphyra purpurea* in the form of a non-hydrolytic

polysaccharide-binding protein [see P. Tomme et al. Cellulose-Binding Domains - Classification and Properties in Enzymatic Degradation of Insoluble Carbohydrates, John N. Saddler and Michael H. Penner (Eds.), ACS Symposium Series, No. 618 (1996)]. However, most of the known CBDs [which are classified and referred to by P. Tomme et al. (*op cit.*) as "cellulose-binding domains"] derive from cellulases and xylanases.

In the present context, the term "cellulose-binding domain" is intended to be understood in the same manner as in the latter reference (P. Tomme et al., *op. cit*), and the abbreviation "CBD" as employed herein will thus often be interpretable either in the broader sense (carbohydrate-binding domain) or in the - in principle - narrower sense (cellulose-binding domain). The P. Tomme et al. reference classifies more than 120 "cellulose-binding domains" into 10 families (I-X) which may have different functions or roles in connection with the mechanism of substrate binding. However, it is anticipated that new family representatives and additional CBD families will appear in the future.

In proteins/polypeptides in which CBDs occur (e.g. enzymes, typically hydrolytic enzymes), a CBD may be located at the N or C terminus or at an internal position.

That part of a polypeptide or protein (e.g. hydrolytic enzyme) which constitutes a CBD *per se* typically consists of more than about 30 and less than about 250 amino acid residues. For example: those CBDs listed and classified in Family I in accordance with P. Tomme et al. (*op. cit.*) consist of 33-37 amino acid residues, those listed and classified in Family IIa consist of 95-108 amino acid residues, those listed and classified in Family VI consist of 85-92 amino acid residues, whilst one CBD (derived from a cellulase from *Clostridium thermocellum*) listed and classified in Family VII consists of 240 amino acid residues. Accordingly, the molecular weight of an amino acid sequence constituting a CBD *per se* will typically be in the range of from about 4kD to about 40kD, and usually below about 35kD.

Enzyme hybrids

Enzyme classification numbers (EC numbers) referred to in the present specification with claims are in accordance with the Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, Academic Press Inc., 1992.

As already indicated to some extent (*vide supra*), modified enzymes as referred to herein (in the following also denoted "enzyme hybrids") include species comprising an amino acid sequence of an amylolytic enzyme [which in the context of the present invention may, e.g., be an α-amylase (EC 3.2.1.1), an isoamylase (EC 3.2.1.68) or a pullulanase (EC 3.2.1.41)] linked (i.e. covalently bound) to an amino acid sequence comprising a CBD.

Other CBD-containing enzyme hybrids of interest in relation to degradation of starch include, e.g., hybrids comprising an amino acid sequence of a glucan 1,4-α-maltohydrolase (EC 3.2.1.133), a β-amylase (EC 3.2.1.2), a glucoamylase (EC 3.2.1.3), or a neopullulanase (EC 3.2.1.135).

CBD-containing enzyme hybrids, as well as detailed descriptions of the preparation and purification thereof, are known in the art [see, e.g., WO 90/00609, WO 94/24158 and WO 95/16782, as well as Greenwood et al., Biotechnology and Bioengineering 44 (1994) pp. 1295-1305]. They may, e.g., be prepared by transforming into a host cell a DNA construct comprising at least a fragment of DNA encoding the cellulose-binding domain ligated, with or without a linker, to a DNA sequence encoding the enzyme of interest, and growing the transformed host cell to express the fused gene. The resulting recombinant product (enzyme hybrid) - often referred to in the art as a "fusion protein - may be described by the following general formula:

A-CBD-MR-X

In the latter formula, A-CBD is the N-terminal or the C-terminal region of an amino acid sequence comprising at least the

carbohydrate-binding domain (CBD) *per se*. MR is the middle region (the "linker"), and X is the sequence of amino acid residues of a polypeptide encoded by a DNA sequence encodng the enzyme (or other protein) to which the CBD is to be linked.

The moiety A may either be absent (such that A-CBD is a CBD *per se*, i.e. comprises no amino acid residues other than those constituting the CBD) or may be a sequence of one or more amino acid residues (functioning as a terminal extension of the CBD *per se*). The linker (MR) may be a bond, or a short linking group comprising from about 2 to about 100 carbon atoms, in particular of from 2 to 40 carbon atoms. However, MR is preferably a sequence of from about 2 to about 100 amino acid residues, more preferably of from 2 to 40 amino acid residues, such as from 2 to 15 amino acid residues.

The moiety X may constitute either the N-terminal or the C-terminal region of the overall enzyme hybrid.

It will thus be apparent from the above that the CBD in an enzyme hybrid of the type in question may be positioned C-terminally, N-terminally or internally in the enzyme hybrid.

Cellulases (cellulase genes) useful for preparation of CBDs

Techniques suitable for isolating a cellulase gene are well known in the art. In the present context, the term "cellulase" refers to an enzyme which catalyses the degradation of cellulose to glucose, cellobiose, triose and/or other cello-oligosaccharides.

Preferred cellulases (i.e. cellulases comprising preferred CBDs) in the present context are microbial cellulases, particularly bacterial or fungal cellulases. Endoglucanases (EC 3.2.1.4), particularly mono-component (recombinant) endoglucanases, are a preferred class of cellulases,.

Useful examples of bacterial cellulases are cellulases derived from or producible by bacteria from the group consisting of *Pseudomonas, Bacillus, Cellulomonas, Clostridium, Microspora, Thermotoga, Caldocellum* and Actinomycets such as *Streptomyces, Termomonospora* and *Acidothemus*, in particular from the group consisting of *Pseudomonas cellulolyticus, Bacillus lautus,*

Bacillus agaradherens, *Cellulomonas fimi*, *Clostridium thermocellum*, *Clostridium stercorarium* *Microspora bispora*, *Termomonospora fusca*, *Termomonospora cellulolyticum* and *Acidothemus cellulolyticus*.

The cellulase may be an acid, a neutral or an alkaline cellulase, i.e. exhibiting maximum cellulolytic activity in the acid, neutral or alkaline range, respectively.

A useful cellulase is an acid cellulase, preferably a fungal acid cellulase, which is derived from or producible by fungi from the group of genera consisting of *Trichoderma*, *Myrothecium*, *Aspergillus*, *Phanaerochaete*, *Neurospora*, *Neocallimastix* and *Botrytis*.

A preferred useful acid cellulase is one derived from or producible by fungi from the group of species consisting of *Trichoderma viride*, *Trichoderma reesei*, *Trichoderma longibrachiatum*, *Myrothecium verrucaria*, *Aspergillus niger*, *Aspergillus oryzae*, *Phanaerochaete chrysosporium*, *Neurospora crassa*, *Neocallimastix partriciarum* and *Botrytis cinerea*.

Another useful cellulase is a neutral or alkaline cellulase, preferably a fungal neutral or alkaline cellulase, which is derived from or producible by fungi from the group of genera consisting of *Aspergillus*, *Penicillium*, *Myceliophthora*, *Humicola*, *Irpex*, *Fusarium*, *Stachybotrys*, *Scopulariopsis*, *Chaetomium*, *Mycogone*, *Verticillium*, *Myrothecium*, *Papulospora*, *Gliocladium*, *Cephalosporium* and *Acremonium*.

A preferred alkaline cellulase is one derived from or producible by fungi from the group of species consisting of *Humicola insolens*, *Fusarium oxysporum*, *Myceliopthora thermophila*, *Penicillium janthinellum* and *Cephalosporium* sp., preferably from the group of species consisting of *Humicola insolens* DSM 1800, *Fusarium oxysporum* DSM 2672, *Myceliopthora thermophila* CBS 117.65, and *Cephalosporium* sp. RYM-202.

A preferred cellulase is an alkaline endoglucanase which is immunologically reactive with an antibody raised against a highly purified ~43kD endoglucanase derived from *Humicola insolens* DSM 1800, or which is a derivative of the latter ~43kD endoglucanase and exhibits cellulase activity.

Other examples of useful cellulases are variants of parent cellulases of fungal or bacterial origin, e.g. a parent cellulase derivable from a strain of a species within one of the fungal genera *Humicola*, *Trichoderma* or *Fusarium*.

Other proteins (protein genes) useful for preparation of CBDs

Examples of other types of hydrolytic enzymes which comprise a CBD are, as already mentioned, xylanases, mannanases, arabinofuranosidases, acetylesterases and chitinases. As also mentioned previously, CBDs have also been found, for example, in certain algae, e.g. in the red alga *Porphyra purpurea* in the form of a non-hydrolytic polysaccharide-binding protein. Reference may be made to P. Tomme et al. (*op cit.*) for further details concerning sources (organism genera and species) of such CBDs. Further CBDs of interest in relation to the present invention include CBDs deriving from glucoamylases (EC 3.2.1.3) or from CGTases (EC 2.4.1.19).

CBDs deriving from such sources will also be generally be suitable for use in the context of the invention. In this connection, techniques suitable for isolating, e.g., xylanase genes, mannanase genes, arabinofuranosidase genes, acetylesterase genes, chitinase genes (and other relevant genes) are well known in the art.

Isolation of a CBD

In order to isolate a cellulose-binding domain of, e.g., a cellulase, several genetic engineering approaches may be used. One method uses restriction enzymes to remove a portion of the gene and then to fuse the remaining gene-vector fragment in frame to obtain a mutated gene that encodes a protein truncated for a particular gene fragment. Another method involves the use of exonucleases such as *Ba*131 to systematically delete nucleotides either externally from the 5' and the 3' ends of the DNA or internally from a restricted gap within the gene. These gene-deletion methods result in a mutated gene encoding a shortened gene molecule whose expression product may then be evaluated for

substrate-binding (e.g. cellulose-binding) ability. Appropriate substrates for evaluating the binding ability include cellulosic materials such as Avicel™ and cotton fibres. Other methods include the use of a selective or specific protease capable of cleaving a CBD, e.g. a terminal CBD, from the remainder of the polypeptide chain of the protein in question

As already indicated (*vide supra*), once a nucleotide sequence encoding the substrate-binding (carbohydrate-binding) region has been identified, either as cDNA or chromosomal DNA, it may then be manipulated in a variety of ways to fuse it to a DNA sequence encoding the enzyme of interest. The DNA fragment encoding the carbohydrate-binding amino acid sequence, and the DNA encoding the enzyme of interest are then ligated with or without a linker. The resulting ligated DNA may then be manipulated in a variety of ways to achieve expression. Preferred microbial expression hosts include certain *Aspergillus* species (e.g. *A. niger* or *A. oryzae*), *Bacillus* species, and organisms such as *Escherichia coli* or *Saccharomyces cerevisiae*.

Amylolytic enzymes

Amylases (in particular α-amylases) which are appropriate as the basis for CBD/amylase hybrids of the types employed in the context of the present invention include those of bacterial or fungal origin. Chemically or genetically modified mutants of such amylases are included in this connection. Relevant α-amylases include, for example, α-amylases obtainable from *Bacillus* species, in particular a special strain of *B. licheniformis*, described in more detail in GB 1296839. Relevant commercially available amylases include Duramyl™, Termamyl™, Fungamyl™ and BAN™ (all available from Novo Nordisk A/S, Bagsvaerd, Denmark), and Rapidase™ and Maxamyl P™ (available from Gist-Brocades, Holland), and Optitherm™ (available from Solvay), and Spezym AA™ and Spezyme Delta AA (available from Genencor), and Keistase™ (available from Daiwa).

Other amylases (in particular α-amylases) which are appropriate as the basis for CBD/amylase hybrids of the types

employed in the context of the present invention include a hybrid α-amylase consisting of 1-35 N-terminal amino acids of BAN™ (available from Novo Nordisk) and the C-terminal 36-483 C-terminal amino acids of Termamyl™ (available from Novo Nordisk) with one or more of the following mutations H156Y, A181T, N190F A209V, Q264S; Termamyl™ with one or more of the following mutations I201E, D207H, E211Q, H205S; or Maxamyl™ (available from Gist-brocades/Genencor), with one or more of the following mutations H133Y, N188P,S.

Starch- or starch-fragment-debranching enzymes

Isoamylases: isoamylases (EC 3.2.1.68) appropriate as the basis for CBD/isoamylase hybrids of the types employed in the context of the present invention include those of bacterial origin. Chemically or genetically modified mutants of such isoamylases are included in this connection. Relevant isoamylases include, for example, isoamylases obtainable from *Pseudomonas* species, (e.g. *Pseudomonas* sp. SMP1 or *P. amyloderomosa* SB15), *Bacillus* species (e.g. *B. amyloliquefaciens*), *Flavobacterium* species or *Cytophaga* (*Lysobacter*) species.

Pullulanases: pullulanases (EC 3.2.1.41) appropriate as the basis for CBD/pullulanase hybrids of the types employed in the context of the present invention include those of bacterial origin. Chemically or genetically modified mutants of such pullulanases are included in this connection. Relevant pullulanases include, for example, pullulanases obtainable from *Bacillus* species (e.g. *B. acidopullulyticus*; such a Promozyme™, from Novo Nordisk A/S).

Plasmids

Preparation of plasmids capable of expressing fusion proteins having the amino acid sequences derived from fragments of more than one polypeptide are well known in the art (see, e.g. WO 90/00609 and WO 95/16782). The expression cassette may be included within a replication system for episomal maintenance in an appropriate cellular host or may be provided without a replication system, where it may become integrated into the host

genome. The DNA may be introduced into the host in accordance with known techniques such as transformation, microinjection or the like.

Once the fused gene has been introduced into the appropriate host, the host may be grown to express the fused gene. Normally it is desirable additionally to add a signal sequence which provides for secretion of the fused gene. Typical examples of useful fused genes are:

Signal sequence -- (pro-peptide) -- carbohydrate-binding domain -- linker -- enzyme of interest, or

Signal sequence -- (pro-peptide) -- enzyme of interest -- linker -- carbohydrate-binding domain,

in which the pro-peptide sequence normally contains 5-25 amino acid residues.

The recombinant product may be glycosylated or non-glycosylated.

Determination of α-amylolytic activity (KNU)

The α-amylolytic activity of an enzyme or enzyme hybrid may be determined using potato starch as substrate. This method is based on the break-down (hydrolysis) of modified potato starch, and the reaction is followed by mixing samples of the starch/enzyme or starch/hybrid enzyme solution with an iodine solution. Initially, a blackish-blue colour is formed, but during the break-down of the starch the blue colour becomes weaker and gradually turns to a reddish-brown. The resulting colour is compared with coloured glass calibration standards.

One Kilo Novo α-Amylase Unit (KNU) is defined as the amount of enzyme (enzyme hybrid) which, under standard conditions (i.e. at 37±0.05°C, 0.0003 M Ca^{2+}, pH 5.6) dextrinizes 5.26 g starch dry substance (Merck Amylum solubile).

Test conditions suitable for evaluating the performance of CBD-containing enzyme hybrids in starch processing

Test conditions (e.g. conditions of pH, temperature, calcium concentration etc.) suitable for testing, e.g., CBD/α-amylase, CBD/isoamylase or CBD/pullulanase enzyme hybrids as described herein will suitably be conditions as already described above in connection with industrial starch conversion processes. Assay methods suitable for determining enzymatic activity under various conditions (e.g. pH, temperature, calcium concentration etc., depending on the nature of the enzyme hybrid) are well known in the art for numerous types of enzymes which are appropriate for linkage to a CBD as described herein, and a person of ordinary skill in the art will readily be able to select assay procedures suitable for evaluating the enzymatic performance of enzyme hybrids as employed in the present context.

The invention also relates to an isolated DNA sequence encoding a hybrid enzyme with amylolytic activity comprising:

(a) a DNA sequence encoding an amylolytic activity;

(b) a DNA sequences encoding a CBD; and

(c) a DNA sequence or fragments thereof encoding the linker sequence shown in SEQ ID no. 21.

It is often a problem of hybrid enzyme comprising an enzyme and a CDB connected via a linker that they are not very stable due to the linker. The inventors have found that when using the linker shown in SEQ ID NO. 21 or essential parts thereof the hybrids are very stable.

The isolated DNA sequence of the invention typically encodes an enzyme with amylolytic activity, such as α-amylase activity, in particular a *Bacillus* α-amylase activity, especially the activity of Termamyl or a variant thereof, or one of the amylolytic activities mentioned above in the section "Amylolytic enzymes". The CBD may be any CBD e.g the CBDs described above in the section "Carbohydrate-binding domains". In a preferred embodiment the CBD is the CBD of the *Bacillus agaradherens* NCIMB No. 40482 alkaline cellulase Cel5A or the CBD-dimer of *Clostridium stercorarium* (NCIMB 11754) XynA..

In a specific embodiment of the invention the isolated DNA sequence is the Termamyl▒-linker-Cel5A-CBD encoded by plasmid pMB492 shown in SEQ ID No. 19.

In a further aspect the invention relates to a DNA construct comprising the isolated DNA sequence of the invention operably linked to one or more control sequences capable of directing the expression of the DNA sequence in a suitable expression host.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the cellulytic enzyme of the invention in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gene, or the *Bacillus pumilus* xylanase or xylosidase gene, the phage Lambda P_R or P_L promoters, or the *E. coli* lac, trp or tac promoters.

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al. (1980) J. Biol. Chem. 255:12073-12080; Alber and Kawasaki (1982) J. Mol. Appl. Gen. 1:419-434) or alcohol dehydrogenase genes (Young et al. (1982) in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al. (1983) Nature 304:652-654) promoters.

To direct the CBD/enzyme hybrid into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the expression vector. The secretory signal sequence is joined to the DNA sequence encoding the enzyme hybrid in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the amylolytic enzyme. The secretory signal sequence may be that normally associated with the amylolytic enzyme or may be from a

gene encoding another secreted protein.

In a preferred embodiment, the expression vector of the invention may comprise a secretory signal sequence substantially identical to the secretory signal encoding sequence of the *Bacillus licheniformis* α-amylase gene, e.g. as described in WO 86/05812.

Also, measures for amplification of the expression may be taken, e.g. by tandem amplification techniques, involving single or double crossing-over, or by multicopy techniques, e.g. as described in US 4,959,316 or WO 91/09129. Alternatively the expression vector may include a temperature sensitive origin of replication, e.g. as described in EP 283,075.

Procedures for ligating DNA sequences encoding the cellulytic enzyme, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for example, Sambrook et al. (1989) supra.

The invention also relates to a recombinant expression vector comprising the DNA construct of the invention, a promoter, and transcriptional and translational stop signals.

It is also an object of the invention to provide a host cell comprising the DNA construct of the invention.

The host cell of the invention, into which the DNA construct or the recombinant expression vector of the invention is to be introduced, may be any cell which is capable of producing the amylolytic enzyme and includes bacteria, yeast, fungi and higher eukaryotic cells.

Examples of bacterial host cells which, on cultivation, are capable of producing the cellulytic enzyme of the invention are grampositive bacteria such as strains of *Bacillus*, in particular a strain of *B. subtilis, B. licheniformis, B. lentus, B. brevis, B. stearothermophilus, B. alkalophilus, B. amyloliquefaciens, B. coagulans, B. circulans, B. lautus, B. megatherium, B. pumilus, B. thuringiensis* or *B. agaradherens*, or strains of *Streptomyces*, in particular a strain of *S. lividans* or *S. murinus*, or gramnegative bacteria such as

Echerichia coli. The transformation of the bacteria may be effected by protoplast transformation or by using competent cells in a manner known *per se* (cf. *Sambrook et al.* (1989) *supra*).

When expressing the CBD/enzyme hybrid in bacteria such as *E. coli*, the enzyme may be retained in the cytoplasm, typically as insoluble granules (known as inclusion bodies), or may be directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed and the granules are recovered and denatured after which the cellulytic enzyme is refolded by diluting the denaturing agent. In the latter case, the hybrid enzyme may be recovered from the periplasmic space by disrupting the cells, e.g. by sonication or osmotic shock, to release the contents of the periplasmic space and recovering the hybrid enzyme.

The transformed or transfected host cell described above is then cultured in a suitable nutrient medium under conditions permitting the expression of the cellulytic enzyme, after which the resulting cellulytic enzyme is recovered from the culture.

The medium used to culture the cells may be any conventional medium suitable for growing the host cells, such as minimal or complex media containing appropriate supplements. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g., in catalogues of the American Type Culture Collection). The cellulytic enzyme produced by the cells may then be recovered from the culture medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g., ammonium sulphate, purification by a variety of chromatographic procedures, e.g., ion exchange chromatography, gelfiltration chromatography, affinity chromatography, or the like, dependent on the type of cellulytic enzyme in question.

The present invention also relates to methods for producing a CBD/enzyme hybrid of the present invention comprising (a) cultivating a *Bacillus* strain to produce a supernatant

comprising the polypeptide; and (b) recovering the polypeptide.

The present invention also relates to methods for producing a hybrid enzyme of the present invention comprising (a) cultivating a host cell under conditions conducive to expression of the polypeptide; and (b) recovering the polypeptide.

In both methods, the cells are cultivated in a nutrient medium suitable for production of the hybrid enzyme using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e.g., references for bacteria and yeast; Bennett, J.W. and LaSure, L., eds. (1991) More Gene Manipulations in Fungi, Academic Press, CA). Suitable media are available from commercial suppliers or may be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it is recovered from cell lysates.

The hybrid enzyme may be detected using methods known in the art that are specific for the hybrid enzymes. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the enzyme. Procedures for determining amylolytic activity are known in the art and are described below.

The resulting hybrid enzyme may be recovered by methods known in the art. For example, the hybrid enzyme may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration,

extraction, spray-drying, evaporation, or precipitation. The recovered hybrid enzyme may then be further purified by a variety of chromatographic procedures, e.g., ion exchange chromatography, gel filtration chromatography, affinity chromatography, or the like.

The hybrid enzyme of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing (IEF), differential solubility (e.g., ammonium sulfate precipitation), or extraction (see, e.g., Protein Purification (Janson and Ryden, eds.), VCH Publishers, New York, 1989).

In a final aspect the invention relates to an isolated and purified CBD/enzyme hybrid encoded by the isolated DNA sequence of the invention, in particular the hybrid shown in SEQ ID No. 20.

MATERIALS AND METHODS

Materials:

Enzymes and enzyme hybrids:

Termamyl▒-linker-CBD_{EGV} : Hybrid of Termamyl▒ and the fungal CBD_{EGV} from *Humicola insolens* EGV. The construction of the hybrid is described in Example 9.

CBD_{CenA}-Termamyl▒ : Hybrid of the CBD_{CenA} from *Cellulomonas fimi* endoglucanase A (CenA) and Termamyl▒ via a linker. The construction of the hybrid is described in Example 8.

Termamyl▒ (available from Novo Nordisk A/S)

Plasmids:

pDN1528 (S.Jørgensen et al. (1991) Journal of Bacteriology, vol. 173, No., p-559-567.)

pBluescriptKSII- (Stratagene, USA).

pDN1981 (P.L. Jørgensen, C.K.Hansen, G.B.Poulsen and B.Diderichsen (1990) In vivo genetic engineering: homologues recombination as a tool for plasmid construction, Gene, 96, p37-41.)

pSJ1678: Described in WO 94/19454; pDN1981: Described by Jørgensen et al. (1990) Gene 96:37-41).

Strains:

Bacillus AC13 NCIMB 40482 (identical to *Bacillus agaradherens* DSM 8721) expressing the endoglucanase enzyme encoding DNA sequence of SEQ ID NO:1.described in Example 1 below

E. coli strain: Cells of *E. coli* SJ2 (Diderichsen et al. (1990) J. Bacteriol. 172:4315-4321), which encodes alpha-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis* were prepared for and transformed by electroporation using a Gene Pulser™ electroporator from BIO-RAD as described by the supplier.

B.subtilis PL2306 was used as the transformation host strain. It is a cellulase-negative strain developed by introducing a disruption in the transcriptional unit of the known *Bacillus subtilis* cellulase gene in *B.subtilis* strain DN1885(Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990) Cloning of *aldB*, which encodes alpha-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis*. J. Bacteriol. 172:4315-4321). Not only was the cellulase gene of DN1885 disrupted but also two protease encoding genes where disrupted, namely the *aprE* (Stahl,M.L. and E.Ferrari 1984 Replacement of the *Bacillus subtilis* subtilisin structural gene with an In vitro-derived deletion mutation. *J.Bacteriol.* 158:411-418) and *nprE* (Yang, M.Y. et al 1984 Cloning of the neutral protease gene of Bacillus subtilis and the use of the cloned gene to create an in vitro-derived deletion mutation. *J.Bacteriol.* 160:16-21) genes

The disruption was performed essentially as described in *Bacillus subtilis* and other Gram-Positive Bacteria; A.L.

Sonenshein, J.A. Hoch and Richard Losick, Eds. American Society for Microbiology, 1993, p.618).

Bacillus subtilis: ToC46 (Diderichsen, B., Wedsted, U., Hedegaard, L., Jensen, B. R., Sjøholm, C. (1990) Cloning of aldB, which encodes alpha-acetolactate decarboxylase, an exoenzyme from Bacillus brevis. J. Bacteriol., 172, 4315-4321) Was used as a secondary expression host, competent cells and transformation was performed as described above.

Solutions/Media/Reagents

Waxy maize from Cerestar

Corn Starch Cerestar (89% DS) GL 03406 Batch 624362

TY and LB agar (as described in Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995).

SB: 32 g Tryptone, 20 g Yeast Extract, 5 g NaCl and 5 ml 1 N NaOH are mixed in sterile water to a final volume of 1 liter. The solution is sterilised by autoclaving for 20 min at 121°C.

10% Avicel: 100 g of Avicel (FLUKA, Switserland) is mixed with sterile water to a final volume of 1 litre, and the 10% Avicel is sterilised by autoclaving for 20 min at 121°C.

Buffer: 0.05 M potassium phosphate, pH 7.5

Methods

DE determination

DE (dextrose equivalent is defined as the amount of reducing carbohydrate (measured as dextrose-equivalents) in a sample expressed as w/w% of the total amount of dissolved dry matter). It is measured by the neocuproine assay (Dygert, Li Floridana(1965) Anal. Biochem. No 368). The principle of the neocuproine assay is that $CuSO_4$ is added to the sample, Cu^{++} is reduced by the reducing sugar and the formed neocuproine

complex is measured at 450 nm.

General molecular biology methods:

DNA manipulations and transformations were performed using standard methods of molecular biology (Sambrook et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor lab., Cold Spring Harbor, NY; Ausubel, F. M. et al. (eds.) "Current protocols in Molecular Biology". John Wiley and Sons, 1995; Harwood, C. R., and Cutting, S. M. (eds.) "Molecular Biological Methods for Bacillus". John Wiley and Sons, 1990).

Enzymes for DNA manipulations were used according to the specifications of the suppliers.

Cellulytic Activity

Cellulytic activity may be measured in cellulase viscosity units (CEVU), determined at pH 9.0 with carboxymethyl cellulose (CMC) as substrate.

Cellulase viscosity units are determined relatively to an enzyme standard (< 1% water, kept in N_2 atmosphere at -20°C; arch standard at -80°C). The standard used, 17-1187, is 4400 CEVU/g under standard incubation conditions, i.e., pH 9.0, Tris Buffer 0.1 M, CMC Hercules 7 LFD substrate 33.3 g/l, 40.0°C for 30 minutes.

α-amylase-Termamyl Activity

See Novo Nordisk analytical method AF 9/6, available on request.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use various constructs and perform the various methods of the present invention and are not intended to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, parts are parts by weight, temperature is in degrees centigrade, and pressure is at or near atmospheric pressure. Efforts have been made to

ensure accuracy with respect to numbers used (e.g., length of DNA sequences, molecular weights, amounts, particular components, etc.), but some deviations should be accounted for.

EXAMPLE 1

Cloning of *Bacillus agaradherens* Endoglucanase Gene

Genomic DNA Preparation.

The strain NCIMB 40482 (identical to *Bacillus agaradherens* DSM 8721) was propagated in liquid medium as described in WO 94/01532. After 16 hours of incubation at 30°C and 300 rpm, the cells were harvested, and genomic DNA was isolated by the method described by Pitcher et al. (1989) Lett. Appl. Microbiol. 8:151-156).

Genomic Library Construction.

Genomic DNA was partially digested with restriction enzyme Sau3A and size-fractionated by electrophoresis on a 0.7 % agarose gel. Fragments of between 2 and 7 kb in size were isolated by electrophoresis onto DEAE-cellulose paper (Dretzen et al. (1981) Anal. Biochem. 112:295-298). Isolated DNA fragments were ligated to BamHI digested, pSJ1678 plasmid DNA.

PCR Amplification.

In order to obtain the endoglucanase gene as ligated to the pSJ1678 vector, the ligation mixture was used as DNA template in a PCR reaction containing 200 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 2.5 mM $MgCl_2$, Expand High Fidelity buffer, 2.0 units of Expand High Fidelity PCR system enzyme mix and 300 nM of each of the following primers:

Primer 1 (#9555):

5'-TCACAGATCCTC-GCGAATTGGTGCGGCCGCGTNGTNG-ARGARCAYGGNC-3' (SEQ ID No. 3).

Primer 1 is a degenerated primer designed to match the amino acid sequence (Val-Val-Glu-Glu-His-Gly-Gln) (SEQ ID No. 4) of

the N-terminal amino acid sequence presented in WO94/01532. The last amino acid is only presented by the first nucleotide of the codon namely C. C is the 3'-nucleotide of the primer.

Furthermore, a NotI site is included at the 5'- end for cloning purposes these nucleotides are underlined. Primer 2 (#9029):

5'-CAGAGCAAGAGATTACGCGC-3' (SEQ ID NO:5).

Primer 2 corresponds to a sequence present in the pSJ1678 vector.

The PCR cycling was performed in a Hans Landgraf THERMOCYCLER (Hans Landgraf, Germany), following the profile:

1 x (120 seconds at 94°C);

10 x (10 seconds at 94°C; 30 seconds at 55°C; 240 seconds at 72°C);

30 x (10 seconds at 94°C; 30 seconds at 55°C; 180 seconds at 72°C; adding 20 seconds to the keep time at 72°C for each new cycle); and

1 x (300 seconds at 72°C).

The PCR product was gel purified by gel eletrophoresis in a 0.7% agarose gel, and the relevant fragment (approx. 1.7 kb) was excised from the gel and purified using QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5.

This DNA was used as a template for a PCR re-amplification using the same primers, mixture and cycle profile as above.

The PCR product was gel purified by gel eletrophoresis in a 0.7% agarose gel, and the relevant fragment was excised from the gel and purified using QIAquick Gel extraction Kit. The purified DNA was eluted in 50 µl of 10 mM Tris-HCl, pH 8.5.

The purified DNA was digested with NotI and HindIII, gel purified as above, and ligated to the vector pBluescriptII KS- (Stratagene, USA), also digested with NotI and HindIII, and the ligation mixture was used to transform *E. coli* SJ2.

Cells were plated on LB agar plates containing ampicillin (200 µg/ml) supplemented with X-gal (5-Bromo-4-chloro-3-indolyl alpha-D-Galactopyranoside, 50 µg/ml).

Identification and Charaterization of Positive Clones.

The transformed cells were plated on LB agar plates containing ampicillin (200 µg/ml) supplemented with X-gal (5-Bromo-4-chloro-3-indolyl alpha-D-Galactopyranoside, 50 µg/ml), and incubated at 37°C overnight. The next day white colonies were rescued by restreaking these onto fresh LB-ampicillin agar plates and incubated at 37°C overnight. The day after, single colonies of each clone were transferred to liquid LB medium containing ampicillin (200 µg/ml), and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit. 5 µl samples of the plasmids are digested with *NotI* and *HindIII*. The digestions were checked by gel electrophoresis on a 0.7 % agarose gel (NuSieve, FMC). The appearance of a DNA fragment of approximately 1.0 kb indicated a positive clone.

Nucleotide Sequencing the Cloned DNA Fragment.

Qiagen purified plasmid DNA was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA) and the primer "Reverse" or the primer "Forward":

Reverse: 5'-GTTTTCC-CAGTCACGAC-3' (SEQ ID No.6),
Forward: 5'-GCGGATAACAATTTCACACAGG-3' (SEQ ID No. 7).

The DNA was sequenced using an Applied Biosystems 373A automated sequencer according to the manufacturers instructions. Analysis of the sequence data is performed according to Devereux et al. (1984) Nucleic Acids Res. 12:387-395).

From this sequence new primers could be designed for performing Inverse PCR [cf. McPherson et al. (eds) in PCR-A practical approach; 1991 IRL Press).

Inverse PCR on Genomic DNA of Strain NCIMB 40482.

Genomic DNA was isolated as described above. 2 mg of pure genomic DNA was digested with EcoRI. The EcoRI was heat inactivated at 65°C for 20 minutes, after which a phenol:chloroform extraction of DNA was performed. DNA was finally ethanol precipitated and resuspended in 20 ml TE.

1 ml of EcoRI digested DNA was ligated with T4-DNA ligase in 100 ml reaction mixture containing T4 ligase buffer and 1 Unit T4-DNA ligase (Boehringer Mannheim, Germany). After 18 hours of ligation at 14°C, the ligase was heat inactivated at 68°C for 10 minutes. In order to linearize the circulized genomic DNA fragments prior to Inverse PCR, the ligation mixture was supplemented with 10 U of BstEII (a BstEII site was present internally of the DNA sequence obtained above).

50 ml of the BstEII digested ligation mixture was used as template in a PCR reaction containing 200 mM of each nucleotide (dATP, dCTP, dGTP and dTTP), 2.5 mM $MgCl_2$, Expand High Fidelity buffer, 2.0 units of Expand High Fidelity PCR system enzyme mix, and 300 nM of each of the following primers:

Primer 3 (#19719): 5'-TGACCCGTACGGTCCGTGGG-3' (SEQ ID No. 8), and

Primer 4 (#19720): 5'-GGCTCTTGATTTTGTGTCCACC-3'(SEQ ID No.9).

The PCR cycling was performed in a Hans Landgraf THERMOCYCLER (Hans Landgraf, Germany), following the profile:

1 x (120 seconds at 94°C);

10 x (10 seconds at 94°C; 30 seconds at 55°C; 240 seconds at 72°C);

30 x (10 seconds at 94°C; 30 seconds at 55°C; 180 seconds at 72°C adding 20 seconds to the keep time at 72°C for each new cycle); and

1 x (300 seconds at 72°C).

The PCR product was gel purified by gel eletrophoresis in a 0.7% agarose gel, and the relevant fragment (approx. 4-5 kb) was excised from the gel and purified using QIAquick Gel extraction Kit. The purified DNA was eluted in 50 µl of 10mM

Tris-HCl, pH 8.5.

Nucleotide Sequencing the Inverse-PCR DNA Fragment.

Qiagen purified DNA was sequenced with the Taq deoxy terminal cycle sequencing kit (Perkin Elmer, USA), and the primer 1, 3 and 4 described above, using an Applied Biosystems 373A automated sequencer according to the manufacturers instructions. Analysis of the sequence data is performed according to Devereux et al. (1984) *supra*). Based upon the obtained sequence two new primers were designed in order to clone the alkaline endoglucanase as presented as SEQ ID No. 12. The primers were #20887 (SEQ ID No. 10) and #100084 (SEQ ID NO. 14) as described below.

EXAMPLE 2

Expression of the Alkaline Endoglucanase in *Bacillus subtilis*

The nucleotide sequence in SEQ ID No. 12 was cloned by PCR for introduction in an expression plasmid pDN1981.

PCR was performed as described below on 500 ng of genomic DNA, using the following two primers containing NdeI and KpnI (the KpnI site is conveniently present in the amplified sequence) restriction sites for introducing the endoglucanase encoding DNA sequence to pDN1981 for expression:

Primer 5 (#20887):

5'-GTA GGC TCA GTC ATA TGT TAC ACA TTG AAA GGG GAG GAG AAT CAT GAA AAA GAT AAC TAC TAT TTT TGT CG-3' (SEQ ID No. 10), and

Primer 7 (#100084):

5'- CCT CGC GAG GTA CCA GCG GCC GCG TAC CAC CAA TTA AGT ATG GTA C -3' (SEQ ID No. 14)

The underlined nucleotides of Primer 5 corresponds to the NdeI site, and the underlined nucleotides in the Primer 7 is part of the KpnI site present in the sequence.

Using the Expand™ Long Template PCR system (available from Boehringer Mannheim, Germany) amplification was performed using

a mixture consisting of (Buffer 1 diluted 10 times) and 200 µM of each dNTP, 2.5 units of Enzyme mix (Boehringer Mannheim, Germany) and 500 pmol of each primer.

The PCR reactions was performed using a DNA Thermal Cycler (available from Landgraf, Germany). One incubation at 94°C for 2 minutes followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 4 minutes. Followed by 25 cycles of PCR performed using a cycle profile of denaturation at 94°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 68°C for 3 minutes (this duration of extension is extended with 20 seconds for each of the 25 cycles).

Aliquots of 10 µl of the amplification product is analysed by electrophoresis in 0.7 % agarose gels (NuSieve, FMC) with ReadyLoad 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

After PCR cycling, the PCR fragment was purified using QIA-quick PCR column Kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5, digested with NdeI and KpnI, and purified and ligated to digested pDN1981. The ligation mixture was used to transform *B. subtilis* PL2304.

Competent cells were prepared and transformed as described by *Yasbin et al.* [*Yasbin R E, Wilson G A & Young F E;* Transformation and transfection in lysogenic strains of *Bacillus subtilis* : evidence for selective induction of prophage in competent cells; J Bacteriol 1975 **121** 296-304].

Isolation and Test of *Bacillus subtilis* Transformants

The transformed cells were plated on LB agar plates containing 10 mg/ml Kanamycin, 0.4% glucose, 10 mM KH2PO4 and 0.1% AZCL HE-cellulose (Megazyme, Australia), and incubated at 37 °C for 18 hours. Endoglucanase positive colonies were identified as colonies surrounded by a blue halo.

Each of the positive transformants were inoculated in 10 ml

TY-medium containing 10 mg/ml Kanamycin. After 1 day of incubation at 37°C and stirring at 250 rpm, 50 ml supernatant was removed. The endoglucanase activity was identified by adding 50 ml supernatant to holes punched in the agar of LB agar plates containing 0.1 % AZCL HE-cellulose.

After 16 hours of incubation at 37 °C, blue halos surrounding holes indicated expression of the endoglucanase in *Bacillus subtilis*.

EXAMPLE 3

Analysis of the Cloned Sequence.

The protein sequence derived from the cloned endoglucanase gene shows an endoglucanase of the following composition:

Amino acid residues 1 to 26 correspond to a signal peptide; amino acid residues 27 to 326 constitute the actual endoglucanase (homologues to other family 5 glycosyl hydrolases); amino acid residues 327 to 354 correspond to a linker; amino acid residues 355 to 400 correspond to a cellulose binding domain (as described in Example 3); amino acid residues 401 to 416 correspond to a linker; and amino acid residues 417 to 462 constitute a second cellulose binding domain (highly homologues to the first one (at amino acid residues 355 to 400)).

The molar extinction coefficient was determined as 146,370. The molecular weight was approximately 52 kD.

For the protein without the signal sequence the molar extinction coefficient was determined as 146.370. The molecular weight was approximately 49 kD.

The enzyme has no cysteine, and the charged amino acids give a calculated pI of around 4.

EXAMPLE 4

Subcloning of a partial Termamyl sequence.

The α-amylase gene encoded on pDN1528 was PCR amplified for introduction of a BamHI site in the 3'-end of the coding region. The PCR and the cloning was done as follows.

Approximately 10 to 20 ng of plasmid pDN1528 was PCR amplified in HiFidelity PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity Expand enzyme mix, and 300 pmol of each primer:

Primer 8, #5289
5'-GCT TTA CGC CCG ATT GCT GAC GCT G -3' (SEQ ID No. 15)

Primer 9, #26748
5'-GCG ATG AGA CGC GCG GCC GCC TAT CTT TGA ACA TAA ATT GAA ACG GAT CCG -3' (SEQ ID No. 16)
Restriction site BamHI are underlined.

The PCR reactions was performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 2 minutes, 30 seconds at 60°C and 45 seconds at 72°C followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds and twenty cycles of denaturation at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds (at this elongation step 20 seconds are added every cycle). 10 µl aliquots of the amplification product was analysed by electrophoresis in 1.0 % agarose gels (NuSieve, FMC) with ReadyLoad 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

40 µl aliquots of the PCR product generated as described above were purified using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment was digested with BamHI and PstI, electrophoresed in 1.0% low gelling temperature agarose (SeaPlaque GTG, FMC) gels, the relevant fragment was excised from the gel, and purified using QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to BamHI-PstI digested pBluescriptII KS- and the ligation mixture was used to

transform *E.coli* SJ2.

Cells were plated on LB agar plates containing ampicillin (200 µg/ml) and supplemented with X-gal (5-Bromo-4-chloro-3-indolyl alpha-D-galactopyranoside, 50 µg/ml), and incubated at 37°C over night. Next day white colonies were re-streaked onto fresh LB-ampicillin agar plates and incubated at 37°C over night. The next day single colonies were transferred to liquid LB medium containing (200 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. 5 µl samples of the plasmids were digested with PstI and BamHI. The digestions were checked by gelelectrophoresis on a 1.0% agarose gel (NuSieve, FMC). One positive clone, containing the PstI-BamHI fragment containing part of the alfa-amylase gene, was designated pMB335. This plasmid was then used in the construction of α-amylase-CBD hybrids.

In vitro amplification of the linker and the most C-terminal CBD of *Bacillus agaradherens* NCIMB No. 40482.

Approximately 100 to 200 ng of chromosomal DNA obtained from *Bacillus agaradherens* NCIMB No. 40482 (as described in the Examples 1 to 3 above) was PCR amplified in HiFidelity▒ PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity▒ Expand enzyme mix, and 300 pmol of each primer:

Primer 10, #110150A

5'- GCT GCA GGA TCC GTT TCA ATT TAT GTT CAA AGA TCT GAT CCA GAT TCA GGA G -3' (SEQ ID No. 17)

Primer 11, #100084

5'-CCT CGC GAG GTA CCA GCG GCC GCG TAC CAC CAA TTA AGT ATG GTA C-3' (SEQ ID NO. 18)

Restriction sites BamHI and NotI are underlined.

The primers were designed to amplify the linker and most C-

terminal CBD of the endoglucanase encoding gene of *Bacillus agaradherens* NCIMB No. 40482 described in the Examples above).

The PCR reaction was performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 2 minutes, 30 seconds at 60°C and 45 seconds at 72°C followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds and twenty cycles of denaturation at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds (at this elongation step 20 seconds are added every cycle). 10 µl aliquots of the amplification product was analysed by electrophoresis in 1.5 % agarose gels (NuSieve, FMC) with ReadyLoad 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

Cloning by polymerase chain reaction (PCR):
Subcloning of PCR fragments.

40 µl aliquots of the PCR products generated as described above were purified using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA was eluted in 50 µl of 10mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment was digested with NotI and partially digested with BamHI, electrophoresed in 1.5% low gelling temperature agarose (SeaPlaque GTG, FMC) gels, the relevant fragment was excised from the gels, and purified using QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment was then ligated to BamHI-NotI digested pMB335 and the ligation mixture was used to transform *E.coli* SJ2.

Identification and characterization of positive clones.

Cells were plated on LB agar plates containing z (200 µg/ml) and incubated at 37°C over night. Next day colonies were restreaked onto fresh LB-ampicillin agar plates and incubated at 37°C over night. The next day single colonies were

transferred to liquid LB medium containing (200 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to the manufacturer's instructions. Five-µl samples of the plasmids were digested with BamHI and NotI. The digestions were checked by gelelectrophoresis on a 1.5% agarose gel (NuSieve, FMC). The appearance of a DNA fragment of the same size as seen from the PCR amplification indicated a positive clone.

One positive clone, containing the fusion construct of the α-amylase gene and the CBD of *Bacillus agaradherens* NCIMB No. 40482 alkaline cellulase Cel5A, was designated MBamyC5ANewlink.

Cloning of the fusion construct into a *Bacillus* based expression vector.

The pDN1528 vector contains the amyL gene of *B. licheniformis* this gene is actively expressed in *B. subtilis* resulting in the production of active α-amylase appearing in the supernatant. For expression purposes the DNA encoding the fusion protein as constructed above was introduced to pDN1528.

This was done by digesting p MBamyC5ANewlink and pDN1528 with SalI-NotI, purifying the fragments and ligating the 4.7 kb pDN1528 SalI-NotI fragment with the 0.5 kb pMBamyC5ANewlink SalI-NotI fragment. This created an inframe fusion of the hybrid construction with the Termamyl gene. See sequence for pMB492 (SEQ ID No. 19).

The ligation mixture was used to transform competent cells of PL2306. Cells were plated on LB agar plates containing chloramphenicol (6 µg/ml), 0.4% glucose and 10mM potassium hydrogen phosphate and incubated at 37°C over night. Next day colonies were restreaked onto fresh LBPG chloramphenicol agar plates and incubated at 37°C over night. The next day single colonies of each clone were transferred to liquid LB medium containing chloramphenicol (6 µg/ml) and incubated overnight at 37°C with shaking at 250 rpm.

Plasmids were extracted from the liquid cultures using QIAgen Plasmid Purification mini kit (Qiagen, USA) according to

the manufacturer's instructions, however the resuspension buffer was supplemented with 1 mg/ml of Chicken Egg White Lysozyme (SIGMA, USA) prior to lysing the cells at 37°C for 15 minutes. 5 µl samples of the plasmids were digested with BamHI and NotI. The digestions were checked by gelelectrophoresis on a 1.5% agarose gel (NuSieve, FMC). The appearance of a DNA fragment of the same size as seen from the PCR amplification indicated a positive clone. One positive clone was designated MB492.

Expression, secretion and functional analysis of the fusion protein.

The clone MB492 (expressing Termamyl▒ fused to *Bacillus agaradherens*-Cel5A-linker-CBD) was incubated for 20 hours in SB-medium at 37°C and 250 rpm. 1 ml of cell-free supernatant was mixed with 200 µl of 10% Avicel. The mixture was left for 1 hour incubation at 0°C. After this binding of CBD to Avicel the Avicel with CBD was spun 5 minutes at 5000g. The pellet was re-suspended in 100 µl of SDS-page buffer, boiled at 95°C for 5 minutes, spun at 5000g for 5 minutes and 25 µl was loaded on a 4-20% Laemmli Tris-Glycine, SDS-PAGE NOVEX gel (Novex, USA). The samples were electrophoresed in a Xcell™ Mini-Cell (NOVEX, USA) as recommended by the manufacturer, all subsequent handling of gels including staining with comassie, destaining and drying were performed as described by the manufacturer.

The appearance of a protein band of approx. 60 kDa, indicated expression in *B.subtilis* of the Termamyl▒-Linker-CBD fusion encoded on the plasmid pMB492 (SEQ ID No. 19). The expression protein sequence of the fusion construction of pMB492 is shown in SEQ ID No. 20.

The linker region of interest as described in this example is the specific sequence:

SDPDSGEPDPTPPSDPG (SEQ ID No. 21)

Example 5

Isolation of genomic DNA from *Clostridium stercorarium* NCIMB 11754.

Clostridium stercorarium NCIMB 11754 was grown anaerobically at 60°C in specified media as recommended by The National Collections of Industrial and Marine Bacteria Ltd. (Scotland). Cells were harvested by centrifugation.

Genomic DNA was isolated as described by Pitcher et al.(Pitcher, D. G., Saunders, N. A., Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol., 8, 151-156).

In vitro amplification of the CBD-dimer of *Clostridium stercorarium* (NCIMB 11754) XynA.

Approximately 100 to 200 ng of genomic DNA (isolated as described above) was PCR amplified in HiFidelity PCR buffer (Boehringer Mannheim, Germany) supplemented with 200 µM of each dNTP, 2.6 units of HiFidelity Expand enzyme mix, and 300 pmol of each primer:

Primer 12, #114135
5'-GCT GCA GGA TCC GTT TCA ATT TAT GTT CAA AGA TCT CCA ACT CCT GCC CCA TCT CAA AGC-3' (SEQ ID NO. 22)

Primer 13, #110151
5'-GCG ATG AGA CGC GCG GCC GCT ACT ACC AGT CAA CAT TAA CAG GAC CTG AG -3' (SEQ ID NO. 23)
Restriction sites BamHI and NotI are underlined.

The primers were designed to amplify the DNA encoding the Cellulose Binding Domain of the XynA encoding gene of *Clostridium stercorarium* (NCIMB 11754), the DNA sequence was extracted from the database GenBank under the accession number D13325.

The PCR reaction was performed using a DNA thermal cycler (Landgraf, Germany). One incubation at 94°C for 2 minutes, 30 seconds at 60°C and 45 seconds at 72°C followed by ten cycles of PCR performed using a cycle profile of denaturation at 94°C

for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds and twenty cycles of denaturation at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds (at this elongation step 20 seconds are added every cycle). 10 µl aliquots of the amplification product was analyzed by electrophoresis in 1.0 % agarose gels (NuSieve, FMC) with ReadyLoad 100bp DNA ladder (GibcoBRL, Denmark) as a size marker.

Cloning by polymerase chain reaction (PCR):
Subcloning of PCR fragments.

40 µl aliquots of the PCR products generated as described above are purified using QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer's instructions. The purified DNA is eluted in 50 µl of 10 mM Tris-HCl, pH 8.5. 25 µl of the purified PCR fragment is digested with BamHI and EagI, electrophoresed in 1.0% low gelling temperature agarose (SeaPlaque GTG, FMC) gels, the relevant fragment is excised from the gels, and purified using QIAquick Gel extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolated DNA fragment is then ligated to BamHI-NotI digested pMB335 and the ligation mixture is used to transform *E.coli* SJ2.

The following steps were then performed as described above:

-Identification and characterisation of positive clones.

-Cloning of the fusion construct into a *Bacillus* based expression vector.

-Expression, secretion and functional analysis of the fusion protein.

The appearance of a protein band of approximately 87 kDa on the comassie stained SDS-PAGE, shows positive expression of the hybrid in *Bacillus subtilis*.

The resulting hybrid is thus expressed in *Bacillus subtilis* clone MBXynCBD2 and is encoded in the DNA sequence SEQ ID No. 24 which can be translated to the protein sequence shown in SEQ ID No. 25.

EXAMPLE 6

CBD_{Cel5A}-linker-Termamyl▒ starch processing

It is investigated whether or not CBD_{Cel5A}-linker-Termamyl▒ (*i.e. Bacillus agaradherens* NCIMB 40482 endoglucanase C-terminal CBD linked to Termamyl▒ via the linker shown in SEQ ID No. 21 constructed as described in Example 4) gives an improved liquefaction of starch per µg enzyme protein/g dry substance compared to Termamyl▒ at pH 6.0 and 40 ppm Ca^{2+}.

A shaking oil bath is heated to 105°C. Two starch slurries (30% DS with 40 ppm Ca^{++}) are prepared, the pH is adjusted to 6.0 with NaOH. CBD_{Cel5A}-linker-Termamyl▒ and Termamyl▒, respectively, are well mixed into the slurries.

From each slurry four portions of 10 g each are taken. Each portion are placed in an Erlenmeyer flask with screw cap. The flasks were placed in the oil bath for 8 minutes at 105°C and then 90 minutes at 95°C.

After 7 minutes and 45 seconds in the oil bath, the thermostat of the oil bath is adjusted to 95.4°C and 2 litre oil at room temperature are added to the oil bath. A clock is started and samples (1 flask of each slurry) are taken after 20, 40, 60, and 90 minutes. 2 drops of 1 N HCl is added to each flask to inactivate the amylase.

The DE-value is then determined as a function of time to compare the starch liquefaction per µg enzyme/g DS of CBD_{Cel5A}-linker-Termamyl▒ with Termamyl▒.

EXAMPLE 7

Construction of the CBD_{CenA} expression vector pCBDT001.

The gene fragment encoding the 103 residue CBD_{CenA} from *Cellulomonas fimi* endoglucanase A (CenA) was cloned in the high expression vector pTugEO7K3. Appropriate restriction sites were introduced at the 5' and 3' ends of the *CBD_{CenA}* gene by PCR. Each PCR mixture (50 ml total volume) contained 25 ng template DNA (pTZ18R-1.6*cenA*; Damude 1995 Doctoral thesis, University of British Columbia. Canada), 25-50 pmole primers (5'SAENH and 3'SAENH), 10 % dimethyl sulfoxide, 0.4 mM 2'-deoxynucleotide

5'-triphosphates, and 1U *Vent* DNA polymerase in "Thermopol" buffer (New England BioLabs). Twenty successive cycles of denaturation at 94°C for 30 seconds, followed by annealing at 55°C for 30 seconds, and primer extension at 72°C for 54 seconds were performed. A *Spe*I site (*underlined*) was introduced at the 5' end of the *CBD*$_{CenA}$ gene fragment, using the oligonucleotide (5'SAENH)

Primer 14

5'-AGGTCTACTAGTCCCGGCTGCCGCGTCGAC-3' (SEQ ID No. 27)

as primer. *Eco*RI (*underlined*), *Nhe*I (*in bold*) and *Hind*III (*in italics*) restriction sites were introduced at the 3' end of the CBD$_{CenA}$ sequence using the oligonucleotide (3'SAENH)

Primer 15

5 '-CCGATTA*AAGCTT*ATTA**GCTAGC**ACGGAATTCCGTGGGGCTGGTCGTCGGCAC-3' (SEQ ID No. 28)

as primer. The resulting 0.38 kb PCR fragment was digested with *Spe*I and *Hind*III and ligated in frame with the Cex leader peptide at the *Nhe*I-*Hind*III site of pTugEO7K3 , previously cut with *Nhe*I and *Hind*III to remove the *CBD*$_{Cex}$ gene fragment. The final construct pCBDT001 was verified by restriction and PCR analysis.

2. Construction of the CBD-Termamyl▒ hybrid expression vector pNAMK 1.0 .

The plasmid pSJ3368 a derivative of pDN1528 (S.Jørgensen et al. (1991) Journal of Bacteriology, vol. 173, No., p-559-567.) containing the *Termamyl*▒ gene, was isolated from *Bacillus* by standard methods. Appropriate restriction sites for recloning the *Termamyl*▒ gene fragment in the *E. coli* vector pCBDT001 and for the construction of the hybrids were introduced by PCR. Each PCR reaction mixture (50 ml total volume) contained 15 ng template DNA (pSJ3368), 3 pmol primers (PAM1 and PAM2), 2 mM $MgSO_4$, 10 % dimethyl sulfoxide, 0.4 mM 2'-deoxynucleotide 5'-

triphosphates and 1U *Vent* DNA polymerase in "Thermopol" buffer (New England BioLabs). Thirty successive cycles were performed as follows: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and primer extension at 72°C for 1.54 min.

A *Nhe*I (*underlined*) and *Nco*I site were introduced at the 5' end of the gene with the oligonucleotide (PAM1)

Primer 16
5'-TCATGAGCCATGGCTAGCGCAAATCTTAATGGGACGCTGATG-3'
(SEQ ID NO. 29)

as primer. An *Spe*I (*in bold*) and *Hin*dIII site (*underlined*) were introduced at the3' end of the Termamyl gene using the oligonucleotide (PAM2)

Primer 17
5'-ATGACTAAGCTTAC TTACTTA*GTGATGGTGATGGTGATG***ACTAGT**TCTTTGAA CATAAATTGAAACCGA-3' (SEQ ID NO. 30)

as primer. This also introduced a His_6-tag (*in italics*) for easy purification of the hybrid protein by immobilized metal affinity chromatography (IMAC), and a stop codon immediately preceding the *Hin*dIII restriction sequence. The resulting 1.5 kb fragment was digested with *Nhe*I and *Hin*dIII and cloned in frame with the CBD_{CenA} at the *Nhe*I-*Hin*dIII site of pCBDT001 to give pNAMK 1.0. The construct was verified by restricion digesting with *Nhe*I and *Hin*dIII and by automated sequencing.

CBD_{CenA}-PTPTTP-Termamyl▒ production and purification

Overnight cultures of *E. coli* JM101, harboring plasmid pNAM1.0, were diluted 500-fold in terrific broth (TB; 12 g tryptone, 24 g yeast extract, 9.8 g K_2HPO_4, 2.2 g KH_2PO_4 and 8 g (10 ml) glycerol in 1l) (Sambrook *et al.*, 1989)(ref: Sambrook J., Fritsch, E.F., & Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.) supplemented with 1.25 mM $CaCl_2$ and 100 mg kanamycin per ml and grown at 30°C to an A_{600}

of 3.0-5.0. Protein production was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The cultures were incubated for an additional 18 hours at 30°C by which time the CBD-Termamyl▒ hybrid had leaked into the culture medium. Cells were removed by centrifugation at 4°C for 10 minutes at 13,000 *x g*. The protein was precipitated from the clarified supernatant with 70% $(NH_4)_2SO_4$ with stirring overnight at 4°C. Proteins were recovered by centrifugation at 11,000 *x g* and the pellet was dissolved in 20 mM Tris-HCl, pH 8.0 (binding buffer). After further centrifugation at 15000 *x g*, the clarified supernatants was loaded onto a Ni^{2+} agarose column (Novagen, Markham, ON). The column was washed with 40 mM imidazole, 200 mM NaCl, 20 mM Tris-HCl, pH 8.0 (wash buffer). Bound proteins were eluted with a gradient of imidazole (0-500 mM) in 20 mM Tris-HCl buffer containing 500 mM NaCl. $CaCl_2$ was immediately added to the fractions to a final concentration of 1 mM to stabilize the protein . Fractions were analysed on SDS-PAGE (12%) and by activity measurements.

The NAM1.0 nucleotide sequence is shown in SEQ ID NO. 31 and can be translated into the amino acid sequence shown in SEQ ID No. 32.

EXAMPLE 8

Termamyl linker fungal CBD from *Humicola insolens* EGV.

pNAMK6.1 (Termamyl▒-linker-CBD_{EGV})

The Termamyl vector NAM 2.0 for C-terminal CBD:

Each PCR reaction mixture (50 ml total volume) contained 15 ng template DNA (pSJ3368), 3 pmol primers (5Term2 and 3Term2), 2 mM $MgSO_4$, 10 % dimethyl sulfoxide, 0.4 mM 2'-deoxynucleotide 5'-triphosphates and 1U *Vent* DNA polymerase in "Thermopol" buffer (New England BioLabs). Thirty successive cycles were performed as follows: denaturation at 95°C for 1 min, annealing at 55°C for 1 min and primer extension at 72°C for 1.54 min.

*Nhe*I (*underlined*) and *EcoRI* (*in bold*) sites were introduced at the 5' end of the *Termamyl* gene with the oligonucleotide (5Term2)

Primer 18

5'-CATATGGCTAGCGAATTCGCAAATCTTAATGGGACGCTG-3' (SEQ ID NO. 33)

as primer. StuI (*underlined*), *Spe*I (*in bold*) and *Hin*dIII sites (*in italics*) were introduced at the3' end of the Termamyl▒ gene using the oligonucleotide (3Term2)

Primer 19

5'-*AAGCTT***ACTAGT**AGGCCTTCTTTGAACATAAATT GAAA-3' (SEQ ID NO. 34)

as primer. The construct was verified by restricion digesting and by automated sequencing.

The fungal CBD vector:

pCBDT006 was obtained by cloning the gene fragment encoding CBD_{EGV} from *Humicola insolens* endoglucanase V (WO 91/17243) in pTugEO7K3. Appropriate restriction sites were introduced at the 5' and 3' ends of the *CBD_{EGV}* gene by PCR. Each PCR mixture (50 ml total volume) contained 25 ng template DNA 25-50 pmole primers (N137 and N1PTcs), 10 % dimethyl sulfoxide, 0.4 mM 2'-deoxynucleotide 5'-triphosphates, and 1U *Vent* DNA polymerase in "Thermopol" buffer (New England BioLabs). Twenty successive cycles of denaturation at 96°C for 45 seconds, followed by annealing at 50°C for 60 seconds, and primer extension at 72°C for 35 seconds were performed. The last cycle was followed by extension at 72°C for 90 seconds.

*Nhe*I (*underlined*), *Eco*RI (*in bold, underlined*), *Stu*I (*in bold*) restriction site were introduced before the artificial linker (*in small letters, italics*), *Spe*I (*in italics, underlined*) and *Eco*47III (*in small, bold*) sites were introduced after the linker at the 3' end of the *CBD_{EGV}* sequence using the oligonucleotide (5CBDT6)

Primer 20

5 '- CCATGGGCTAGCCCT**GAATTC**AGGCCT*ccaacccccACTAGT*CCG**agcgct**CCC AGCGGCTGCACTGCTG -3' (SEQ ID No. 35)

as primer. A *Hin*dIII (*underlined*) restriction site was introduced at the 3' end of the *CBD$_{EGV}$* sequence using the oligonucleotide (3CBDT6)

Primer 21

5'- AGCCTAAGCTTACAGGCACTGATGGTACCAGT -3' (SEQ ID No. 36)

as primer. The resulting 0.18 kb PCR fragment was digested with *Nhe*I and *Hin*dIII and ligated in frame with the Cex leader peptide at the *Nhe*I-*Hin*dIII site of pTugEO7K3 , previously cut with *Nhe*I and *Hin*dIII to remove the *CBD$_{Cex}$* gene fragment. The final construct pCBDT006 was verified by restriction and PCR analysis.

Construction of the hybrid NAMK6.1 (Termamyl▒-linker-CBD$_{EGV}$)

The Termamyl▒ vector NAM2.0 was digested with *Nhe*I and *Stu*I and the resulting 1.48 kb fragment was gel purified using the Gene Clean (Bio101) kit and ligated in frame with the CBD$_{EGV}$ encoding fragment in pCBDT006, previously cut with *Nhe*I and *Stu*I to give pNAMK6.1.

The product has the following characterization MW 60863. Total 537 amino acid residues. First the Termamyl▒ catalytic amylase then the linker in one letter codes:

RPPTPTSPSAPS (SEQ ID No. 37) and finally 38 residues from the fungal CBD. Complete nucleotide Sequence for pNAMK6.1 (pTugK with Termamyl▒-CBD$_{EGV}$ insert) is shown in SEQ ID No. 26.

Example 9

Termamyl▒-linker-CBD$_{EGV}$ starch processing

It was investigated whether or not the Termamyl▒-linker-CBD$_{EGV}$ (Termamyl▒ linker fungal CBD from *Humicola insolens* EGV constructed as described in Example 9 above) gives a better liquefaction of starch per µg enzyme protein/g dry substance compared to Termamyl▒ at pH 6.0 and 40 ppm Ca^{2+}.

A shaking oil bath was heated to 105°C. Three starch slurries (30% DS with 40 ppm Ca^{++}) were prepared, the pH was

adjusted to 6.0 with NaOH. The enzyme was well mixed into the slurries according to the scheme:

Slurry 1: Termamyl▒-linker-CBD_{EGV} 10.9 µg/g DS starch

Slurry 2: Termamyl▒-linker-CBD_{EGV} 8.72 µg/g DS starch

Slurry 3: Termamyl▒ 10.9 µg/g DS starch

From each slurry four portions of 10 g each were taken. Each portion were placed in an Erlenmeyer flask with screw cap. The flasks were placed in the oil bath for 8 minutes at 105°C and then 90 minutes at 95°C.

After 7 minutes and 45 seconds in the oil bath, the thermostat of the oil bath was adjusted to 95.4°C and 2 litre oil at room temperature were added to the oil bath. A clock was started and samples (1 flask of each slurry) were taken after 20, 40, 60, and 90 minutes. 2 drops of 1N HCl was added to each flask to inactivate the amylase.

DE-determinations as function of time:

Minutes	Termamyl▒-linker-CBD_{EGV} 10.9 µg/g DS	Termamyl▒-linker-CBD_{EGV} 8.72 µg/g DS	Termamyl▒ 10.9 µg/g DS
20	6.1	5.6	5.3
40	9.2	7.4	7.7
60	11.6	10.2	9.1
90	14.6	13.4	12.2

As can be seen from the Table above the Termamyl▒-linker-CBD_{EGV} gives a improved liquefaction per µg enzyme/g DS compared to Termamyl▒.

Example 10

CBD_{CenA}-Termamyl▒ starch processing

It was investigate whether or not CBD_{CenA}-Termamyl® (*Cellolumonas fimi* endoglucanase A CBD and Termamyl® via a linker as described in Example 8 above) gives an improved liquefaction of starch per activity unit/g dry substance compared to Termamyl® at pH 6.0 and 40 ppm Ca^{2+}.

A shaking oil bath was heated to 105°C. Two starch slurries (30% DS with 40 ppm Ca^{++}) were prepared, the pH was adjusted to 6.0 with NaOH. The enzyme was well mixed to the slurries according to the scheme:

Slurry 1: CBD_{CenA}-Termamyl® 75NU/g DS starch
Slurry 2: Termamyl® 75NU/g DS starch

From each slurry four portions of 10 g each were taken. Each portion were placed in an Erlenmeyer flask with screw cap. The flasks were placed in the oil bath for 8 minutes at 105°C and then 90 minutes at 95°C.

After 7 minutes and 45 seconds in the oil bath, the thermostat of the oil bath was adjusted to 95.4°C and 2 litre oil at room temperature were added to the oil bath. A clock was started and samples (1 flask of each slurry) were taken after 20, 40, 60, and 90 minutes. 2 drops of 1N HCl were added to each flask to inactivate the amylase.

DE-determinations as function of time:

Minutes	CBD_{CenA}-Termamyl® 75NU/g DS	Termamyl® 75NU/g DS
20	6.1	3.9
40	8.6	6.0
60	12.0	7.7
90	15.4	10.3

As can be seen from the Table above the CBD_{CenA}-Termamyl® gives a better liquefaction per activity unit/g DS compared to Termamyl®.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Allé
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45 4444 8888
(H) TELEFAX: +45 4449 3256
(ii) TITLE OF INVENTION: Hybrid enzymes/Starch processing
(iii) NUMBER OF SEQUENCES: 37
(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1203 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus agaradherens
(B) STRAIN: AC13
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1203
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
ATG AAA AAG ATA ACT ACT ATT TTT GTC GTA TTG CTT ATG ACA GTG GCG        48
Met Lys Lys Ile Thr Thr Ile Phe Val Val Leu Leu Met Thr Val Ala
 1               5                  10                  15

TTG TTC AGT ATA GGA AAC ACG ACT GCT GCT GAT AAT GAT TCA GTT GTA        96
Leu Phe Ser Ile Gly Asn Thr Thr Ala Ala Asp Asn Asp Ser Val Val
            20                  25                  30

GAA GAA CAT GGG CAA TTA AGT ATT AGT AAC GGT GAA TTA GTC AAT GAA       144
Glu Glu His Gly Gln Leu Ser Ile Ser Asn Gly Glu Leu Val Asn Glu
        35                  40                  45

CGA GGC GAA CAA GTT CAG TTA AAA GGG ATG AGT TCC CAT GGT TTG CAA       192
Arg Gly Glu Gln Val Gln Leu Lys Gly Met Ser Ser His Gly Leu Gln
    50                  55                  60

TGG TAC GGT CAA TTT GTA AAC TAT GAA AGT ATG AAA TGG CTA AGA GAT       240
Trp Tyr Gly Gln Phe Val Asn Tyr Glu Ser Met Lys Trp Leu Arg Asp
65                  70                  75                  80

GAT TGG GGA ATA AAT GTA TTC CGA GCA GCA ATG TAT ACC TCT TCA GGA       288
Asp Trp Gly Ile Asn Val Phe Arg Ala Ala Met Tyr Thr Ser Ser Gly
                85                  90                  95

GGA TAT ATT GAT GAT CCA TCA GTA AAG GAA AAA GTA AAA GAG GCT GTT       336
Gly Tyr Ile Asp Asp Pro Ser Val Lys Glu Lys Val Lys Glu Ala Val
            100                 105                 110

GAA GCT GCG ATA GAC CTT GAT ATA TAT GTG ATC ATT GAT TGG CAT ATC       384
Glu Ala Ala Ile Asp Leu Asp Ile Tyr Val Ile Ile Asp Trp His Ile
        115                 120                 125

CTT TCA GAC AAT GAC CCA AAT ATA TAT AAA GAA GAA GCG AAG GAT TTC       432
Leu Ser Asp Asn Asp Pro Asn Ile Tyr Lys Glu Glu Ala Lys Asp Phe
    130                 135                 140
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TTT GAT GAA ATG TCA GAG TTG TAT GGA GAC TAT CCG AAT GTG ATA TAC        480
Phe Asp Glu Met Ser Glu Leu Tyr Gly Asp Tyr Pro Asn Val Ile Tyr
145                 150                 155                 160

GAA ATT GCA AAT GAA CCG AAT GGT AGT GAT GTT ACG TGG GGC AAT CAA        528
Glu Ile Ala Asn Glu Pro Asn Gly Ser Asp Val Thr Trp Gly Asn Gln
                165                 170                 175

ATA AAA CCG TAT GCA GAG GAA GTC ATT CCG ATT ATT CGT AAC AAT GAC        576
Ile Lys Pro Tyr Ala Glu Glu Val Ile Pro Ile Ile Arg Asn Asn Asp
            180                 185                 190

CCT AAT AAC ATT ATT ATT GTA GGT ACA GGT ACA TGG AGT CAG GAT GTC        624
Pro Asn Asn Ile Ile Ile Val Gly Thr Gly Thr Trp Ser Gln Asp Val
        195                 200                 205

CAT CAT GCA GCT GAT AAT CAG CTT GCA GAT CCT AAC GTC ATG TAT GCA        672
His His Ala Ala Asp Asn Gln Leu Ala Asp Pro Asn Val Met Tyr Ala
    210                 215                 220

TTT CAT TTT TAT GCA GGG ACA CAT GGT CAA AAT TTA CGA GAC CAA GTA        720
Phe His Phe Tyr Ala Gly Thr His Gly Gln Asn Leu Arg Asp Gln Val
225                 230                 235                 240

GAT TAT GCA TTA GAT CAA GGA GCA GCG ATA TTT GTT AGT GAA TGG GGA        768
Asp Tyr Ala Leu Asp Gln Gly Ala Ala Ile Phe Val Ser Glu Trp Gly
                245                 250                 255

ACA AGT GCA GCT ACA GGT GAT GGT GGC GTG TTT TTA GAT GAA GCA CAA        816
Thr Ser Ala Ala Thr Gly Asp Gly Gly Val Phe Leu Asp Glu Ala Gln
            260                 265                 270

GTG TGG ATT GAC TTT ATG GAT GAA AGA AAT TTA AGC TGG GCC AAC TGG        864
Val Trp Ile Asp Phe Met Asp Glu Arg Asn Leu Ser Trp Ala Asn Trp
        275                 280                 285


TCT CTA ACG CAT AAA GAT GAG TCA TCT GCA GCG TTA ATG CCA GGT GCA        912
Ser Leu Thr His Lys Asp Glu Ser Ser Ala Ala Leu Met Pro Gly Ala
    290                 295                 300

AAT CCA ACT GGT GGT TGG ACA GAG GCT GAA CTA TCT CCA TCT GGT ACA        960
Asn Pro Thr Gly Gly Trp Thr Glu Ala Glu Leu Ser Pro Ser Gly Thr
305                 310                 315                 320

TTT GTG AGG GAA AAA ATA AGA GAA TCA GCA TCT ATT CCG CCA AGC GAT       1008
Phe Val Arg Glu Lys Ile Arg Glu Ser Ala Ser Ile Pro Pro Ser Asp
                325                 330                 335

CCA ACA CCG CCA TCT GAT CCA GGA GAA CCG GAT CCA ACG CCC CCA AGT       1056
Pro Thr Pro Pro Ser Asp Pro Gly Glu Pro Asp Pro Thr Pro Pro Ser
            340                 345                 350

GAT CCA GGA GAG TAT CCA GCA TGG GAT CCA AAT CAA ATT TAC ACA AAT       1104
Asp Pro Gly Glu Tyr Pro Ala Trp Asp Pro Asn Gln Ile Tyr Thr Asn
        355                 360                 365

GAA ATT GTG TAC CAT AAC GGC CAG CTA TGG CAA GCA AAA TGG TGG ACA       1152
Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr
    370                 375                 380

CAA AAT CAA GAG CCA GGT GAC CCG TAC GGT CCG TGG GAA CCA CTC AAT       1200
Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn
385                 390                 395                 400

TAA                                                                  1203
```

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 400 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Lys Ile Thr Thr Ile Phe Val Val Leu Leu Met Thr Val Ala
 1               5                  10                  15

Leu Phe Ser Ile Gly Asn Thr Thr Ala Ala Asp Asn Asp Ser Val Val
            20                  25                  30

Glu Glu His Gly Gln Leu Ser Ile Ser Asn Gly Glu Leu Val Asn Glu
        35                  40                  45

Arg Gly Glu Gln Val Gln Leu Lys Gly Met Ser Ser His Gly Leu Gln
    50                  55                  60

Trp Tyr Gly Gln Phe Val Asn Tyr Glu Ser Met Lys Trp Leu Arg Asp
65                  70                  75                  80

Asp Trp Gly Ile Asn Val Phe Arg Ala Ala Met Tyr Thr Ser Ser Gly
                85                  90                  95

Gly Tyr Ile Asp Asp Pro Ser Val Lys Glu Lys Val Lys Glu Ala Val
            100                 105                 110

Glu Ala Ala Ile Asp Leu Asp Ile Tyr Val Ile Ile Asp Trp His Ile
        115                 120                 125

Leu Ser Asp Asn Asp Pro Asn Ile Tyr Lys Glu Glu Ala Lys Asp Phe
    130                 135                 140

Phe Asp Glu Met Ser Glu Leu Tyr Gly Asp Tyr Pro Asn Val Ile Tyr
145                 150                 155                 160

Glu Ile Ala Asn Glu Pro Asn Gly Ser Asp Val Thr Trp Gly Asn Gln
                165                 170                 175

Ile Lys Pro Tyr Ala Glu Glu Val Ile Pro Ile Ile Arg Asn Asn Asp
            180                 185                 190

Pro Asn Asn Ile Ile Ile Val Gly Thr Gly Thr Trp Ser Gln Asp Val
        195                 200                 205

His His Ala Ala Asp Asn Gln Leu Ala Asp Pro Asn Val Met Tyr Ala
    210                 215                 220

Phe His Phe Tyr Ala Gly Thr His Gly Gln Asn Leu Arg Asp Gln Val
225                 230                 235                 240

Asp Tyr Ala Leu Asp Gln Gly Ala Ala Ile Phe Val Ser Glu Trp Gly
                245                 250                 255

Thr Ser Ala Ala Thr Gly Asp Gly Gly Val Phe Leu Asp Glu Ala Gln
            260                 265                 270

Val Trp Ile Asp Phe Met Asp Glu Arg Asn Leu Ser Trp Ala Asn Trp
        275                 280                 285

Ser Leu Thr His Lys Asp Glu Ser Ser Ala Ala Leu Met Pro Gly Ala
    290                 295                 300

Asn Pro Thr Gly Gly Trp Thr Glu Ala Glu Leu Ser Pro Ser Gly Thr
305                 310                 315                 320
```

Phe Val Arg Glu Lys Ile Arg Glu Ser Ala Ser Ile Pro Pro Ser Asp
325 330 335

Pro Thr Pro Pro Ser Asp Pro Gly Glu Pro Asp Pro Thr Pro Pro Ser
340 345 350

Asp Pro Gly Glu Tyr Pro Ala Trp Asp Pro Asn Gln Ile Tyr Thr Asn
355 360 365

Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr
370 375 380

Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn
385 390 395 400

(2) INFORMATION FOR SEQ ID NO:3:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 1 (#9555)"
(ix) FEATURE:
(A) NAME/KEY: misc-feature
(B) LOCATION: 33,36,39,42,45,48
(D): OTHER INFORMATION: /Note N= A,G,C or T
R= G or A
Y= C or T
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TCACAGATCC TCGCGAATTG GTGCGGCCGC GTNGTNGARG ARCAYGGNC 49

(2) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Val Val Glu Glu His Gly Gln
5

(2) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 2"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGAGCAAGAG ATTACGCGC 19

(2) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:

(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Reverse Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTTTTCCCAG TCACGAC 17

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Forward Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGATAACA ATTTCACACA GG 22

(2) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 3, #19719"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGACCCGTAC GGTCCGTGGG 20

(2) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 4, #19720"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGCTCTTGAT TTTGTGTCCA CC 22

(2) INFORMATION FOR SEQ ID NO:10:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 5. #20887"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTAGGCTCAG TCATATGTTA CACATTGAAA GGGGAGGAGA ATCATGAAAA AGATAACTAC 60
TATTTTTGTC G 71

(2) INFORMATION FOR SEQ ID NO:11:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 6"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTACCTCGCG GGTACCAAGC GGCCGCTTAA TTGAGTGGTT CCCACGGACC G 51

(2) INFORMATION FOR SEQ ID NO: 12:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1386 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: DNA (genomic)
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Bacillus agaradherens
(B) STRAIN: AC13
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1386
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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ATG AAA AAG ATA ACT ACT ATT TTT GTC GTA TTG CTT ATG ACA GTG GCG        48
Met Lys Lys Ile Thr Thr Ile Phe Val Val Leu Leu Met Thr Val Ala
 1               5                  10                  15

TTG TTC AGT ATA GGA AAC ACG ACT GCT GCT GAT AAT GAT TCA GTT GTA        96
Leu Phe Ser Ile Gly Asn Thr Thr Ala Ala Asp Asn Asp Ser Val Val
            20                  25                  30

GAA GAA CAT GGG CAA TTA AGT ATT AGT AAC GGT GAA TTA GTC AAT GAA       144
Glu Glu His Gly Gln Leu Ser Ile Ser Asn Gly Glu Leu Val Asn Glu
        35                  40                  45

CGA GGC GAA CAA GTT CAG TTA AAA GGG ATG AGT TCC CAT GGT TTG CAA       192
Arg Gly Glu Gln Val Gln Leu Lys Gly Met Ser Ser His Gly Leu Gln
    50                  55                  60

TGG TAC GGT CAA TTT GTA AAC TAT GAA AGT ATG AAA TGG CTA AGA GAT       240
Trp Tyr Gly Gln Phe Val Asn Tyr Glu Ser Met Lys Trp Leu Arg Asp
65                  70                  75                  80

GAT TGG GGA ATA AAT GTA TTC CGA GCA GCA ATG TAT ACC TCT TCA GGA       288
Asp Trp Gly Ile Asn Val Phe Arg Ala Ala Met Tyr Thr Ser Ser Gly
                85                  90                  95

GGA TAT ATT GAT GAT CCA TCA GTA AAG GAA AAA GTA AAA GAG GCT GTT       336
Gly Tyr Ile Asp Asp Pro Ser Val Lys Glu Lys Val Lys Glu Ala Val
            100                 105                 110

GAA GCT GCG ATA GAC CTT GAT ATA TAT GTG ATC ATT GAT TGG CAT ATC       384
Glu Ala Ala Ile Asp Leu Asp Ile Tyr Val Ile Ile Asp Trp His Ile
        115                 120                 125

CTT TCA GAC AAT GAC CCA AAT ATA TAT AAA GAA GAA GCG AAG GAT TTC       432
Leu Ser Asp Asn Asp Pro Asn Ile Tyr Lys Glu Glu Ala Lys Asp Phe
    130                 135                 140

TTT GAT GAA ATG TCA GAG TTG TAT GGA GAC TAT CCG AAT GTG ATA TAC       480
Phe Asp Glu Met Ser Glu Leu Tyr Gly Asp Tyr Pro Asn Val Ile Tyr
145                 150                 155                 160

GAA ATT GCA AAT GAA CCG AAT GGT AGT GAT GTT ACG TGG GGC AAT CAA       528
Glu Ile Ala Asn Glu Pro Asn Gly Ser Asp Val Thr Trp Gly Asn Gln
                165                 170                 175
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ATA AAA CCG TAT GCA GAG GAA GTC ATT CCG ATT ATT CGT AAC AAT GAC        576
Ile Lys Pro Tyr Ala Glu Glu Val Ile Pro Ile Ile Arg Asn Asn Asp
            180                 185                 190

CCT AAT AAC ATT ATT ATT GTA GGT ACA GGT ACA TGG AGT CAG GAT GTC        624
Pro Asn Asn Ile Ile Ile Val Gly Thr Gly Thr Trp Ser Gln Asp Val
        195                 200                 205

CAT CAT GCA GCT GAT AAT CAG CTT GCA GAT CCT AAC GTC ATG TAT GCA        672
His His Ala Ala Asp Asn Gln Leu Ala Asp Pro Asn Val Met Tyr Ala
    210                 215                 220

TTT CAT TTT TAT GCA GGG ACA CAT GGT CAA AAT TTA CGA GAC CAA GTA        720
Phe His Phe Tyr Ala Gly Thr His Gly Gln Asn Leu Arg Asp Gln Val
225                 230                 235                 240

GAT TAT GCA TTA GAT CAA GGA GCA GCG ATA TTT GTT AGT GAA TGG GGA        768
Asp Tyr Ala Leu Asp Gln Gly Ala Ala Ile Phe Val Ser Glu Trp Gly
                245                 250                 255

ACA AGT GCA GCT ACA GGT GAT GGT GGC GTG TTT TTA GAT GAA GCA CAA        816
Thr Ser Ala Ala Thr Gly Asp Gly Gly Val Phe Leu Asp Glu Ala Gln
            260                 265                 270

GTG TGG ATT GAC TTT ATG GAT GAA AGA AAT TTA AGC TGG GCC AAC TGG        864
Val Trp Ile Asp Phe Met Asp Glu Arg Asn Leu Ser Trp Ala Asn Trp
        275                 280                 285

TCT CTA ACG CAT AAA GAT GAG TCA TCT GCA GCG TTA ATG CCA GGT GCA        912
Ser Leu Thr His Lys Asp Glu Ser Ser Ala Ala Leu Met Pro Gly Ala
    290                 295                 300

AAT CCA ACT GGT GGT TGG ACA GAG GCT GAA CTA TCT CCA TCT GGT ACA        960
Asn Pro Thr Gly Gly Trp Thr Glu Ala Glu Leu Ser Pro Ser Gly Thr
305                 310                 315                 320

TTT GTG AGG GAA AAA ATA AGA GAA TCA GCA TCT ATT CCG CCA AGC GAT       1008
Phe Val Arg Glu Lys Ile Arg Glu Ser Ala Ser Ile Pro Pro Ser Asp
                325                 330                 335

CCA ACA CCG CCA TCT GAT CCA GGA GAA CCG GAT CCA ACG CCC CCA AGT       1056
Pro Thr Pro Pro Ser Asp Pro Gly Glu Pro Asp Pro Thr Pro Pro Ser
            340                 345                 350

GAT CCA GGA AAG TAT CCA GCA TGG GAT CCA AAT CAA ATT TAC ACA AAT       1104
Asp Pro Gly Lys Tyr Pro Ala Trp Asp Pro Asn Gln Ile Tyr Thr Asn
        355                 360                 365

GAA ATT GTG TAC CAT AAC GGC CAG CTA TGG CAA GCA AAA TGG TGG ACA       1152
Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr
    370                 375                 380

CAA AAT CAA GAG CCA GGT GAC CCG TAC GGT CCG TGG GAA CCA CTC AAA       1200
Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Lys
385                 390                 395                 400

TCT GAT CCA GAT TCA GGA GAA CCG GAT CCA ACG CCC CCA AGT GAT CCA       1248
Ser Asp Pro Asp Ser Gly Glu Pro Asp Pro Thr Pro Pro Ser Asp Pro
                405                 410                 415

GGA GAA TAT CCA GCA TGG GAC CCA ACG CAA ATT TAC ACA GAT GAA ATT       1296
Gly Glu Tyr Pro Ala Trp Asp Pro Thr Gln Ile Tyr Thr Asp Glu Ile
            420                 425                 430

GTG TAC CAT AAC GGC CAG CTA TGG CAA GCC AAA TGG TGG ACA CAA AAT       1344
Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr Gln Asn
        435                 440                 445
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CAA GAG CCA GGT GAC CCA TAC GGT CCG TGG GAA CCA CTC AAT                1386
Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn
    450                 455                 460
```

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 462 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
Met Lys Lys Ile Thr Thr Ile Phe Val Val Leu Leu Met Thr Val Ala
 1               5                  10                  15

Leu Phe Ser Ile Gly Asn Thr Thr Ala Ala Asp Asn Asp Ser Val Val
            20                  25                  30

Glu Glu His Gly Gln Leu Ser Ile Ser Asn Gly Glu Leu Val Asn Glu
        35                  40                  45

Arg Gly Glu Gln Val Gln Leu Lys Gly Met Ser Ser His Gly Leu Gln
    50                  55                  60

Trp Tyr Gly Gln Phe Val Asn Tyr Glu Ser Met Lys Trp Leu Arg Asp
65                  70                  75                  80

Asp Trp Gly Ile Asn Val Phe Arg Ala Ala Met Tyr Thr Ser Ser Gly
                85                  90                  95

Gly Tyr Ile Asp Asp Pro Ser Val Lys Glu Lys Val Lys Glu Ala Val
            100                 105                 110

Glu Ala Ala Ile Asp Leu Asp Ile Tyr Val Ile Ile Asp Trp His Ile
        115                 120                 125

Leu Ser Asp Asn Asp Pro Asn Ile Tyr Lys Glu Glu Ala Lys Asp Phe
    130                 135                 140

Phe Asp Glu Met Ser Glu Leu Tyr Gly Asp Tyr Pro Asn Val Ile Tyr
145                 150                 155                 160

Glu Ile Ala Asn Glu Pro Asn Gly Ser Asp Val Thr Trp Gly Asn Gln
                165                 170                 175

Ile Lys Pro Tyr Ala Glu Glu Val Ile Pro Ile Ile Arg Asn Asn Asp
            180                 185                 190

Pro Asn Asn Ile Ile Ile Val Gly Thr Gly Thr Trp Ser Gln Asp Val
        195                 200                 205

His His Ala Ala Asp Asn Gln Leu Ala Asp Pro Asn Val Met Tyr Ala
    210                 215                 220

Phe His Phe Tyr Ala Gly Thr His Gly Gln Asn Leu Arg Asp Gln Val
225                 230                 235                 240

Asp Tyr Ala Leu Asp Gln Gly Ala Ala Ile Phe Val Ser Glu Trp Gly
                245                 250                 255

Thr Ser Ala Ala Thr Gly Asp Gly Gly Val Phe Leu Asp Glu Ala Gln
            260                 265                 270

Val Trp Ile Asp Phe Met Asp Glu Arg Asn Leu Ser Trp Ala Asn Trp
        275                 280                 285

Ser Leu Thr His Lys Asp Glu Ser Ser Ala Ala Leu Met Pro Gly Ala
```

290 295 300

Asn Pro Thr Gly Gly Trp Thr Glu Ala Glu Leu Ser Pro Ser Gly Thr
305 310 315 320

Phe Val Arg Glu Lys Ile Arg Glu Ser Ala Ser Ile Pro Pro Ser Asp
325 330 335

Pro Thr Pro Pro Ser Asp Pro Gly Glu Pro Asp Pro Thr Pro Pro Ser
340 345 350

Asp Pro Gly Lys Tyr Pro Ala Trp Asp Pro Asn Gln Ile Tyr Thr Asn
355 360 365

Glu Ile Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr
370 375 380

Gln Asn Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Lys
385 390 395 400

Ser Asp Pro Asp Ser Gly Glu Pro Asp Pro Thr Pro Pro Ser Asp Pro
405 410 415

Gly Glu Tyr Pro Ala Trp Asp Pro Thr Gln Ile Tyr Thr Asp Glu Ile
420 425 430

Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr Gln Asn
435 440 445

Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn
450 455 460

(2) INFORMATION FOR SEQ ID NO: 14:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 7, #100084"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCTCGCGAGG TACCAGCGGC CGCGTACCAC CAATTAAGTA TGGTAC 46

(2) INFORMATION FOR SEQ ID NO: 15:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 8, #5289"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GCTTTACGCC CGATTGCTGA CGCTG 35

(2) INFORMATION FOR SEQ ID NO: 16:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 9, #26748"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GCGATGAGAC GCGCGGCCGC CTATCTTTGA ACATAAATTG AAACGGATCC G 51

(2) INFORMATION FOR SEQ ID NO: 17:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 10, #110150A"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GCTGCAGGAT CCGTTTCAAT TTATGTTCAA AGATCTGATC CAGATTCAGG AG 52

(2) INFORMATION FOR SEQ ID NO: 18:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 11, #100084"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

CCTCGCGAGG TACCAGCGGC CGCGTACCAC CAATTAAGTA TGGTAC 46

(2) INFORMATION FOR SEQ ID NO: 19:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1725 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Hybrid"
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1725
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

```
ATG AAA CAA CAA AAA CGG CTT TAC GCC CGA TTG CTG ACG CTG TTA TTT        48
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
  1               5                  10                  15

GCG CTC ATC TTC TTG CTG CCT CAT TCT GCA GCA GCG GCG GCA AAT CTT        96
Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
             20                  25                  30

AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC AAT GAC GGC       144
Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
         35                  40                  45

CAA CAT TGG AAG CGT TTG CAA AAC GAC TCG GCA TAT TTG GCT GAA CAC       192
Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
     50                  55                  60

GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA ACG AGC CAA       240
Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
```

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65                  70                  75                  80

GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA GGG GAG TTT      288
Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
                85                  90                  95

CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA GGA GAG CTG      336
His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
            100                 105                 110

CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATT AAC GTT TAC GGG      384
Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
        115                 120                 125

GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC GAA GAT GTA      432
Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
    130                 135                 140

ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA ATC TCA GGA      480
Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
145                 150                 155                 160

GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG GGG GCC GGC      528
Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Ala Gly
                165                 170                 175

AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT GAC GGA ACC      576
Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
            180                 185                 190

GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG TTT CAA GGA      624
Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
        195                 200                 205

AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC TAT GAT TAT      672
Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
    210                 215                 220

TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC GCA GCA GAA      720
Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
225                 230                 235                 240

ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA TTG GAC GGA      768
Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
                245                 250                 255

AAC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT TTG CGG GAT      816
Asn Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
            260                 265                 270

TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG TTT ACG GTA      864
Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
        275                 280                 285

GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC TAT TTG AAC      912
Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
    290                 295                 300

AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT CAT TAT CAG      960
Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
305                 310                 315                 320

TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG AGG AAA TTG     1008
Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
                325                 330                 335

CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG GTT ACA TTT     1056
Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
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            340                 345                 350

GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG TCG ACT GTC      1104
Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
        355                 360                 365

CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC ACA AGG GAA      1152
Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
    370                 375                 380

TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG ACG AAA GGA      1200
Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
385                 390                 395                 400

GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT GAA CCG ATC      1248
Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
                405                 410                 415

TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT GAT TAT TTC      1296
Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
            420                 425                 430

GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC AGC TCG GTT      1344
Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
        435                 440                 445

GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC GGT GGG GCA      1392
Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
    450                 455                 460

AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA TGG CAT GAC      1440
Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
465                 470                 475                 480

ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG GAA GGC TGG      1488
Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
                485                 490                 495

GGA GAG TTT CAC GTA AAC GGC GGA TCC GTT TCA ATT TAT GTT CAA AGA      1536
Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
            500                 505                 510

TCT GAT CCA GAT TCA GGA GAA CCG GAT CCA ACG CCC CCA AGT GAT CCA      1584
Ser Asp Pro Asp Ser Gly Glu Pro Asp Pro Thr Pro Pro Ser Asp Pro
        515                 520                 525

GGA GAA TAT CCA GCA TGG GAC CCA ACG CAA ATT TAC ACA GAT GAA ATT      1632
Gly Glu Tyr Pro Ala Trp Asp Pro Thr Gln Ile Tyr Thr Asp Glu Ile
    530                 535                 540

GTG TAC CAT AAC GGC CAG CTA TGG CAA GCC AAA TGG TGG ACA CAA AAT      1680
Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr Gln Asn
545                 550                 555                 560

CAA GAG CCA GGT GAC CCA TAC GGT CCG TGG GAA CCA CTC AAT TAA          1725
Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn  *
                565                 570                 575
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(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 575 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

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Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
  1               5                  10                  15
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Ala Leu Il Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
20 25 30

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
35 40 45

Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
50 55 60

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
65 70 75 80

Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
85 90 95

His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
100 105 110

Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
115 120 125

Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
130 135 140

Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
145 150 155 160

Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Ala Gly
165 170 175

Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
180 185 190

Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
195 200 205

Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
210 215 220

Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
225 230 235 240

Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
245 250 255

Asn Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
260 265 270

Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
275 280 285

Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
290 295 300

Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
305 310 315 320

Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
325 330 335

Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
340 345 350

Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
355 360 365

Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu

370 375 380

Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
385 390 395 400

Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
405 410 415

Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
420 425 430

Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
435 440 445

Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
450 455 460

Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
465 470 475 480

Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
485 490 495

Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
500 505 510

Ser Asp Pro Asp Ser Gly Glu Pro Asp Pro Thr Pro Pro Ser Asp Pro
515 520 525

Gly Glu Tyr Pro Ala Trp Asp Pro Thr Gln Ile Tyr Thr Asp Glu Ile
530 535 540

Val Tyr His Asn Gly Gln Leu Trp Gln Ala Lys Trp Trp Thr Gln Asn
545 550 555 560

Gln Glu Pro Gly Asp Pro Tyr Gly Pro Trp Glu Pro Leu Asn *
565 570 575

2) INFORMATION FOR SEQ ID NO:21:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(ix) FEATURE:
(a) NAME/KEY: misc-feature
(d) OTHER INFORMATION: /desc = "Linker"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser Asp Pro Asp Ser Gly Glu Pro Asp Pro Thr Pro Pro Ser Asp Pro Gly
5 10 15

(2) INFORMATION FOR SEQ ID NO: 22:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 60 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 12, #114135"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

GCTGCAGGAT CCGTTTCAAT TTATGTTCAA AGATCTCCAA CTCCTGCCCC ATCTCAAAGC 60

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 50 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 13, #110151"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCGATGAGAC GCGCGGCCGC TACTACCAGT CAACATTAAC AGGACCTGAG 50

(2) INFORMATION FOR SEQ ID NO: 24:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2346 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Hybrid"
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..2346
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

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ATG AAA CAA CAA AAA CGG CTT TAC GCC CGA TTG CTG ACG CTG TTA TTT        48
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
 1               5                  10                  15

GCG CTC ATC TTC TTG CTG CCT CAT TCT GCA GCA GCG GCG GCA AAT CTT        96
Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
            20                  25                  30

AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC AAT GAC GGC       144
Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
        35                  40                  45

CAA CAT TGG AAG CGT TTG CAA AAC GAC TCG GCA TAT TTG GCT GAA CAC       192
Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
    50                  55                  60

GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT AAG GGA ACG AGC CAA       240
Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
65                  70                  75                  80

GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA GGG GAG TTT       288
Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
                85                  90                  95

CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA AAA GGA GAG CTG       336
His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
            100                 105                 110

CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC ATT AAC GTT TAC GGG       384
Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
        115                 120                 125

GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT GCG ACC GAA GAT GTA       432
Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
    130                 135                 140

ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC CGC GTA ATC TCA GGA       480
Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
145                 150                 155                 160

GAA CAC CTA ATT AAA GCC TGG ACA CAT TTT CAT TTT CCG GGG GCC GGC       528
Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Ala Gly
                165                 170                 175
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AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC CAT TTT GAC GGA ACC      576
Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
            180                 185                 190

GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC TAT AAG TTT CAA GGA      624
Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
        195                 200                 205

AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC GGC AAC TAT GAT TAT      672
Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
    210                 215                 220

TTG ATG TAT GCC GAC ATC GAT TAT GAC CAT CCT GAT GTC GCA GCA GAA      720
Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
225                 230                 235                 240

ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA CTG CAA TTG GAC GGA      768
Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
                245                 250                 255

AAC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT TCT TTT TTG CGG GAT      816
Asn Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
            260                 265                 270

TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG GAA ATG TTT ACG GTA      864
Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
        275                 280                 285

GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG GAA AAC TAT TTG AAC      912
Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
    290                 295                 300

AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG CCG CTT CAT TAT CAG      960
Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
305                 310                 315                 320

TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT GAT ATG AGG AAA TTG     1008
Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
                325                 330                 335

CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG AAA TCG GTT ACA TTT     1056
Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
            340                 345                 350

GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG CTT GAG TCG ACT GTC     1104
Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
        355                 360                 365

CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT ATT CTC ACA AGG GAA     1152
Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
    370                 375                 380

TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG TAC GGG ACG AAA GGA     1200
Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
385                 390                 395                 400

GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC AAA ATT GAA CCG ATC     1248
Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
                405                 410                 415

TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA CAG CAT GAT TAT TTC     1296
Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
            420                 425                 430

GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA GGC GAC AGC TCG GTT     1344
Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
        435                 440                 445
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GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC GGA CCC GGT GGG GCA      1392
Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
    450                 455                 460

AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT GAG ACA TGG CAT GAC      1440
Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
465                 470                 475                 480

ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC AAT TCG GAA GGC TGG      1488
Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
                485                 490                 495

GGA GAG TTT CAC GTA AAC GGC GGA TCC GTT TCA ATT TAT GTT CAA AGA      1536
Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
            500                 505                 510

TCT CCA ACT CCT GCC CCA TCT CAA AGC CCA ATT AGA AGA GAT GCA TTT      1584
Ser Pro Thr Pro Ala Pro Ser Gln Ser Pro Ile Arg Arg Asp Ala Phe
        515                 520                 525

TCA ATA ATC GAA GCG GAA GAA TAT AAC AGC ACA AAT TCC TCC ACT TTA      1632
Ser Ile Ile Glu Ala Glu Glu Tyr Asn Ser Thr Asn Ser Ser Thr Leu
    530                 535                 540

CAA GTG ATT GGA ACG CCA AAT AAT GGC AGA GGA ATT GGT TAT ATT GAA      1680
Gln Val Ile Gly Thr Pro Asn Asn Gly Arg Gly Ile Gly Tyr Ile Glu
545                 550                 555                 560

AAT GGT AAT ACC GTA ACT TAC AGC AAT ATA GAT TTT GGT AGT GGT GCA      1728
Asn Gly Asn Thr Val Thr Tyr Ser Asn Ile Asp Phe Gly Ser Gly Ala
                565                 570                 575

ACA GGG TTC TCT GCA ACT GTT GCA ACG GAG GTT AAT ACC TCA ATT CAA      1776
Thr Gly Phe Ser Ala Thr Val Ala Thr Glu Val Asn Thr Ser Ile Gln
            580                 585                 590

ATC CGT TCT GAC AGT CCT ACC GGA ACT CTA CTT GGT ACC TTA TAT GTA      1824
Ile Arg Ser Asp Ser Pro Thr Gly Thr Leu Leu Gly Thr Leu Tyr Val
        595                 600                 605

AGT TCT ACC GGC AGC TGG AAT ACA TAT CAA ACC GTA TCT ACA AAC ATC      1872
Ser Ser Thr Gly Ser Trp Asn Thr Tyr Gln Thr Val Ser Thr Asn Ile
    610                 615                 620

AGC AAA ATT ACC GGC GTT CAT GAT ATT GTA TTG GTA TTC TCA GGT CCA      1920
Ser Lys Ile Thr Gly Val His Asp Ile Val Leu Val Phe Ser Gly Pro
625                 630                 635                 640

GTC AAT GTG GAC AAC TTC ATA TTT AGC AGA AGT TCA CCA GTG CCT GCA      1968
Val Asn Val Asp Asn Phe Ile Phe Ser Arg Ser Ser Pro Val Pro Ala
                645                 650                 655

CCT GGT GAT AAC ACA AGA GAC GCA TAT TCT ATC ATT CAG GCC GAG GAT      2016
Pro Gly Asp Asn Thr Arg Asp Ala Tyr Ser Ile Ile Gln Ala Glu Asp
            660                 665                 670

TAT GAC AGC AGT TAT GGT CCC AAC CTT CAA ATC TTT AGC TTA CCA GGT      2064
Tyr Asp Ser Ser Tyr Gly Pro Asn Leu Gln Ile Phe Ser Leu Pro Gly
        675                 680                 685

GGT GGC AGC GCC ATT GGC TAT ATT GAA AAT GGT TAT TCC ACT ACC TAT      2112
Gly Gly Ser Ala Ile Gly Tyr Ile Glu Asn Gly Tyr Ser Thr Thr Tyr
    690                 695                 700

AAA AAT ATT GAT TTT GGT GAC GGC GCA ACG TCC GTA ACA GCA AGA GTA      2160
Lys Asn Ile Asp Phe Gly Asp Gly Ala Thr Ser Val Thr Ala Arg Val
705                 710                 715                 720
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GCT ACC CAG AAT GCT ACT ACC ATT CAG GTA AGA TTG GGA AGT CCA TCG      2208
Ala Thr Gln Asn Ala Thr Thr Ile Gln Val Arg Leu Gly Ser Pro Ser
                725                 730                 735

GGT ACA TTA CTT GGA ACA ATT TAC GTG GGG TCC ACA GGA AGC TTT GAT      2256
Gly Thr Leu Leu Gly Thr Ile Tyr Val Gly Ser Thr Gly Ser Phe Asp
            740                 745                 750

ACT TAT AGG GAT GTA TCC GCT ACC ATT AGT AAT ACT GCG GGT GTA AAA      2304
Thr Tyr Arg Asp Val Ser Ala Thr Ile Ser Asn Thr Ala Gly Val Lys
        755                 760                 765

GAT ATT GTT CTT GTA TTC TCA GGT CCT GTT AAT GTT GAC TGG              2346
Asp Ile Val Leu Val Phe Ser Gly Pro Val Asn Val Asp Trp
    770                 775                 780
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(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 782 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

```
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
 1               5                  10                  15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
            20                  25                  30

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
        35                  40                  45

Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His
    50                  55                  60

Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln
65                  70                  75                  80

Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe
                85                  90                  95

His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu
            100                 105                 110

Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly
        115                 120                 125

Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val
    130                 135                 140

Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly
145                 150                 155                 160

Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Ala Gly
                165                 170                 175

Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr
            180                 185                 190

Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly
        195                 200                 205

Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr
    210                 215                 220
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Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu
225                 230                 235                 240

Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly
                245                 250                 255

Asn Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp
            260                 265                 270

Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val
        275                 280                 285

Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn
    290                 295                 300

Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln
305                 310                 315                 320

Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu
                325                 330                 335

Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe
            340                 345                 350

Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val
        355                 360                 365

Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu
    370                 375                 380

Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly
385                 390                 395                 400

Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile
                405                 410                 415

Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe
            420                 425                 430

Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val
        435                 440                 445

Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala
    450                 455                 460

Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp
465                 470                 475                 480

Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp
                485                 490                 495

Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
            500                 505                 510

Ser Pro Thr Pro Ala Pro Ser Gln Ser Pro Ile Arg Arg Asp Ala Phe
        515                 520                 525

Ser Ile Ile Glu Ala Glu Glu Tyr Asn Ser Thr Asn Ser Ser Thr Leu
    530                 535                 540

Gln Val Ile Gly Thr Pro Asn Asn Gly Arg Gly Ile Gly Tyr Ile Glu
545                 550                 555                 560

Asn Gly Asn Thr Val Thr Tyr Ser Asn Ile Asp Phe Gly Ser Gly Ala
                565                 570                 575

Thr Gly Phe Ser Ala Thr Val Ala Thr Glu Val Asn Thr Ser Ile Gln
            580                 585                 590
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```
Ile Arg Ser Asp Ser Pro Thr Gly Thr Leu Leu Gly Thr Leu Tyr Val
        595                 600                 605

Ser Ser Thr Gly Ser Trp Asn Thr Tyr Gln Thr Val Ser Thr Asn Ile
    610                 615                 620

Ser Lys Ile Thr Gly Val His Asp Ile Val Leu Val Phe Ser Gly Pro
625                 630                 635                 640

Val Asn Val Asp Asn Phe Ile Phe Ser Arg Ser Ser Pro Val Pro Ala
                645                 650                 655

Pro Gly Asp Asn Thr Arg Asp Ala Tyr Ser Ile Ile Gln Ala Glu Asp
            660                 665                 670

Tyr Asp Ser Ser Tyr Gly Pro Asn Leu Gln Ile Phe Ser Leu Pro Gly
        675                 680                 685

Gly Gly Ser Ala Ile Gly Tyr Ile Glu Asn Gly Tyr Ser Thr Thr Tyr
    690                 695                 700

Lys Asn Ile Asp Phe Gly Asp Gly Ala Thr Ser Val Thr Ala Arg Val
705                 710                 715                 720

Ala Thr Gln Asn Ala Thr Thr Ile Gln Val Arg Leu Gly Ser Pro Ser
                725                 730                 735

Gly Thr Leu Leu Gly Thr Ile Tyr Val Gly Ser Thr Gly Ser Phe Asp
            740                 745                 750

Thr Tyr Arg Asp Val Ser Ala Thr Ile Ser Asn Thr Ala Gly Val Lys
        755                 760                 765

Asp Ile Val Leu Val Phe Ser Gly Pro Val Asn Val Asp Trp
    770                 775                 780
```

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6136 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

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TTTGACAGCT TATCATCGAC TGCACGGTGC ACCAATGCTT CTGGCGTCAG GCAGCCATCG      60

GAAGCTGTGG TATGGCTGTG CAGGTCGTAA ATCACTGCAT AATTCGTGTC GCTCAAGGCG     120

CACTCCCGTT CTGGATAATG TTTTTTGCGC CGACATCATA ACGGTTCTGG CAAATATTCT     180

GAAATGAGCT GTTGACAATT AATCATCGGC TCGTATAATG TGTGGAATTG TGAGCGGATA     240

ACAATTTCAC ACAGGAAACA GAATTGATCC ATAACTAACT AATCTAGTAA TAATTTTGTT     300

TAACTTTAAG AAGGAGATAT ATCCATGGAT CCTAGGACCA CGCCCGCACC CGGCCACCCG     360

GCCCGCGGCG CCCGCACCGC TCTGCGCACG ACGCTCGCCG CCGCGGCGGC GACGCTCGTC     420

GTCGGCGCCA CGGTCGTGCT GCCCGCCCAG GCCGCTAGCG AATTCGCAAA TCTTAATGGG     480

ACGCTGATGC AGTATTTTGA ATGGTACATG CCCAATGACG GCCAACATTG GAGGCGTTTG     540

CAAAACGACT CGGCATATTT GGCTGAACAC GGTATTACTG CCGTCTGGAT TCCCCCGGCA     600

TATAAGGGAA CGAGCCAAGC GGATGTGGGC TACGGTGCTT ACGACCTTTA TGATTTAGGG     660
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GAGTTTCATC AAAAAGGGAC GGTTCGGACA AAGTACGGCA CAAAAGGAGA GCTGCAATCT 720

GCGATCAAAA GTCTTCATTC CCGCGACATT AACGTTTACG GGGATGTGGT CATCAACCAC 780

AAAGGCGGCG CTGATGCGAC CGAAGATGTA ACCGCGGTTG AAGTCGATCC CGCTGACCGC 840

AACCGCGTAA TCTCAGGAGA ACACCTAATT AAAGCCTGGA CACATTTTCA TTTTCCGGGG 900

CGCGGCAGCA CATACAGCGA TTTTAAATGG CATTGGTACC ATTTTGACGG AACCGATTGG 960

GACGAGTCCC GAAAGCTGAA CCGCATCTAT AAGTTTCAAG GAAAGGCTTG GGATTGGGAA 1020

GTTTCCAATG AAAACGGCAA CTATGATTAT TTGATGTATG CCGACATCGA TTATGACCAT 1080

CCTGATGTCG CAGCAGAAAT TAAGAGATGG GGCACTTGGT ATGCCAATGA ACTGCAATTG 1140

GACGGTTTCC GTCTTGATGC TGTCAAACAC ATTAAATTTT CTTTTTTGCG GGATTGGGTT 1200

AATCATGTCA GGGAAAAAAC GGGGAAGGAA ATGTTTACGG TAGCTGAATA TTGGCAGAAT 1260

GACTTGGGCG CGCTGGAAAA CTATTTGAAC AAAACAAATT TTAATCATTC AGTGTTTGAC 1320

GTGCCGCTTC ATTATCAGTT CCATGCTGCA TCGACACAGG GAGGCGGCTA TGATATGAGG 1380

AAATTGCTGA ACGGTACGGT CGTTTCCAAG CATCCGTTGA AATCGGTTAC ATTTGTCGAT 1440

AACCATGATA CACAGCCGGG GCAATCGCTT GAGTCGACTG TCCAAACATG GTTTAAGCCG 1500

CTTGCTTACG CTTTTATTCT CACAAGGGAA TCTGGATACC CTCAGGTTTT CTACGGGGAT 1560

ATGTACGGGA CGAAAGGAGA CTCCCAGCGC GAAATTCCTG CCTTGAAACA CAAAATTGAA 1620

CCGATCTTAA AAGCGAGAAA ACAGTATGCG TACGGAGCAC AGCATGATTA TTTCGACCAC 1680

CATGACATTG TCGGCTGGAC AAGGGAAGGC GACAGCTCGG TTGCAAATTC AGGTTTGGCG 1740

GCATTAATAA CAGACGGACC CGGTGGGGCA AAGCGAATGT ATGTCGGCCG GCAAAACGCC 1800

GGTGAGACAT GGCATGACAT TACCGGAAAC CGTTCGGAGC CGGTTGTCAT CAATTCGGAA 1860

GGCTGGGGAG AGTTTCACGT AAACGGCGGG TCGGTTTCAA TTTATGTTCA AAGAAGGCCT 1920

CCAACCCCCA CTAGTCCGAG CGCTCCCAGC GGCTGCACTG CTGAGAGGTG GGCTCAGTGC 1980

GGCGGCAATG GCTGGAGCGG CTGCACCACC TGCGTCGCTG GCAGCACTTG CACGAAGATT 2040

AATGACTGGT ACCATCAGTG CCTGTAAGCT TATTATATTA CTAATTAATT GGGGACCCTA 2100

GAGGTCCCCT TTTTTATTTT AGCTTCACGC TGCCGCAAGC ACTCAGGGCG CAAGGGCTGC 2160

TAAAGGAAGC GGAACACGTA GAAAGCCAGT CCGCAGAAAC GGTGCTGACC CCGGATGAAT 2220

GTCAGCTACT GGGCTATCTG GACAAGGGAA AACGCAAGCG CAAAGAGAAA GCAGGTAGCT 2280

TGCAGTGGGC TTACATGGCG ATAGCTAGAC TGGGCGGTTT TATGGACAGC AAGCGAACCG 2340

GAATTGCCAG CTGGGGCGCC CTCTGGTAAG GTTGGGAAGC CCTGCAAAGT AAACTGGATG 2400

GCTTTCTTGC CGCCAAGGAT CTGATGGCGC AGGGGATCAA GATCTGATCA AGAGACAGGA 2460

TGAGGATCGT TTCGCATGAT TGAACAAGAT GGATTGCACG CAGGTTCTCC GGCCGCTTGG 2520

GTGGAGAGGC TATTCGGCTA TGACTGGGCA CAACAGACAA TCGGCTGCTC TGATGCCGCC 2580

GTGTTCCGGC TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG TCAAGACCGA CCTGTCCGGT 2640

GCCCTGAATG AACTGCAGGA CGAGGCAGCG CGGCTATCGT GGCTGGCCAC GACGGGCGTT 2700

CCTTGCGCAG CTGTGCTCGA CGTTGTCACT GAAGCGGGAA GGGACTGGCT GCTATTGGGC 2760

GAAGTGCCGG GGCAGGATCT CCTGTCATCT CACCTTGCTC CTGCCGAGAA AGTATCCATC 2820

ATGGCTGATG CAATGCGGCG GCTGCATACG CTTGATCCGG CTACCTGCCC ATTCGACCAC 2880

CAAGCGAAAC ATCGCATCGA GCGAGCACGT ACTCGGATGG AAGCCGGTCT TGTCGATCAG 2940

GATGATCTGG ACGAAGAGCA TCAGGGGCTC GCGCCAGCCG AACTGTTCGC CAGGCTCAAG 3000

GCGCGCATGC CCGACGGCGA GGATCTCGTC GTGACACATG GCGATGCCTG CTTGCCGAAT 3060

ATCATGGTGG AAAATGGCCG CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG 3120

GACCGCTATC AGGACATAGC GTTGGCTACC CGTGATATTG CTGAAGAGCT TGGCGGCGAA 3180

TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC CCGATTCGCA GCGCATCGCC 3240

TTCTATCGCC TTCTTGACGA GTTCTTCTGA GCGGGACTCT GGGGTTCGAA ATGACCGACC 3300

AAGCGACGCC CAACCTGCCA TCACGAGATT TCGATTCCAC CGCCGCCTTC TATGAAAGGT 3360

TGGGCTTCGG AATCGTTTTC CGGGACGCCG GCTGGATGAT CCTCCAGCGC GGGGATCTCA 3420

TGCTGGAGTT CTTCGCCCAC CCCAAAAGGA TCTAGGTGAA GATCCTTTTT GATAATCTCA 3480

TGACCAAAAT CCCTTAACGT GAGTTTTCGT TCCACTGAGC GTCAGACCCC GTAGAAAAGA 3540

TCAAAGGATC TTCTTGAGAT CCTTTTTTTC TGCGCGTAAT CTGCTGCTTG CAAACAAAAA 3600

AACCACCGCT ACCAGCGGTG GTTTGTTTGC CGGATCAAGA GCTACCAACT CTTTTTCCGA 3660

AGGTAACTGG CTTCAGCAGA GCGCAGATAC CAAATACTGT CCTTCTAGTG TAGCCGTAGT 3720

TAGGCCACCA CTTCAAGAAC TCTGTAGCAC CGCCTACATA CCTCGCTCTG CTAATCCTGT 3780

TACCAGTGGC TGCTGCCAGT GGCGATAAGT CGTGTCTTAC CGGGTTGGAC TCAAGACGAT 3840

AGTTACCGGA TAAGGCGCAG CGGTCGGGCT GAACGGGGGG TTCGTGCACA CAGCCCAGCT 3900

TGGAGCGAAC GACCTACACC GAACTGAGAT ACCTACAGCG TGAGCTATGA GAAAGCGCCA 3960

CGCTTCCCGA AGGGAGAAAG GCGGACAGGT ATCCGGTAAG CGGCAGGGTC GGAACAGGAG 4020

AGCGCACGAG GGAGCTTCCA GGGGGAAACG CCTGGTATCT TTATAGTCCT GTCGGGTTTC 4080

GCCACCTCTG ACTTGAGCGT CGATTTTTGT GATGCTCGTC AGGGGGGCGG AGCCTATGGA 4140

AAAACGCCAG CAACGCGGCC TTTTTACGGT TCCTGGCCTT TTGCTGGCCT TTTGCTCACA 4200

TGTTCTTTCC TGCGTTATCC CCTGATTCTG TGGATAACCG TATTACCGCC TTTGAGTGAG 4260

CTGATACCGC TCGCCGCAGC CGAACGACCG AGCGCAGCGA GTCAGTGAGC GAGGAAGCGG 4320

AAGAGCGCCT GATGCGGTAT TTTCTCCTTA CGCATCTGTG CGGTATTTCA CACCGCATAT 4380

GCAGATATTT TGTTAAAATT CGCGTTAAAT TTTTGTTAAA TCAGCTCATT TTTTAACCAA 4440

TAGGCCGAAA TCGGCAAAAT CCCTTATAAA TCAAAAGAAT AGACCGAGAT AGGGTTGAGT 4500

GTTGTTCCAG TTTGGAACAA GAGTCCACTA TTAAAGAACG TGGACTCCAA CGTCAAAGGG 4560

CGAAAAACCG TCTATCAGGG CGATGGCCCA CTACGTGAAC CATCACCCTA ATCAAGTTTT 4620

TTGGGGTCGA GGTGCCGTAA AGCACTAAAT CGGAACCCTA AAGGGAGCCC CCGATTTAGA 4680

GCTTGACGGG GAAAGCCGGC GAACGTGGCG AGAAAGGAAG GGAAGAAAGC GAAAGGAGCG 4740

GGCGCTAGGG CGCTGGCAAG TGTAGCGGTC ACGCTGCGCG TAACCACCAC ACCCGCCGCG 4800

CTTAATGCGC CGCTACAGGG CGCGTCAGGT GGCACTTTTC GGGGAAATGT GCGCGGAACC 4860

CCTATTTGTT TATTTTTCTA AATACATTCA AATATGTATC CGCTCATGAG ACAATAACCC 4920

TGCTGCATTT ACGTTGACAC CATCGAATGG TGCAAAACCT TTCGCGGTAT GGCATGATAG 4980

CGCCCGGAAG AGAGTCAATT CAGGGTGGTG AATGTGAAAC CAGTAACGTT ATACGATGTC 5040

GCAGAGTATG CCGGTGTCTC TTATCAGACC GTTTCCCGCG TGGTGAACCA GGCCAGCCAC 5100

GTTTCTGCGA AAACGCGGGA AAAAGTGGAA GCGGCGATGG CGGAGCTGAA TTACATTCCC 5160

AACCGCGTGG CACAACAACT GGCGGGCAAA CAGTCGTTGC TGATTGGCGT TGCCACCTCC 5220

AGTCTGGCCC TGCACGCGCC GTCGCAAATT GTCGCGGCGA TTAAATCTCG CGCCGATCAA 5280

CTGGGTGCCA GCGTGGTGGT GTCGATGGTA GAACGAAGCG GCGTCGAAGC CTGTAAAGCG 5340

GCGGTGCACA ATCTTCTCGC GCAACGCGTC AGTGGGCTGA TCATTAACTA TCCGCTGGAT 5400

GACCAGGATG CCATTGCTGT GGAAGCTGCC TGCACTAATG TTCCGGCGTT ATTTCTTGAT 5460

GTCTCTGACC AGACACCCAT CAACAGTATT ATTTTCTCCC ATGAAGACGG TACGCGACTG 5520

GGCGTGGAGC ATCTGGTCGC ATTGGGTCAC CAGCAAATCG CGCTGTTAGC GGGCCCATTA 5580

AGTTCTGTCT CGGCGCGTCT GCGTCTGGCT GGCTGGCATA AATATCTCAC TCGCAATCAA 5640

ATTCAGCCGA TAGCGGAACG GGAAGGCGAC TGGAGTGCCA TGTCCGGTTT TCAACAAACC 5700

ATGCAAATGC TGAATGAGGG CATCGTTCCC ACTGCGATGC TGGTTGCCAA CGATCAGATG 5760

GCGCTGGGCG CAATGCGCGC CATTACCGAG TCCGGGCTGC GCGTTGGTGC GGATATCTCG 5820

GTAGTGGGAT ACGACGATAC CGAAGACAGC TCATGTTATA TCCCGCCGTT AACCACCATC 5880

AAACAGGATT TTCGCCTGCT GGGGCAAACC AGCGTGGACC GCTTGCTGCA ACTCTCTCAG 5940

GGCCAGGCGG TGAAGGGCAA TCAGCTGTTG CCCGTCTCAC TGGTGAAAAG AAAAACCACC 6000

CTGGCGCCCA ATACGCAAAC CGCCTCTCCC CGCGCGTTGG CCGATTCATT AATGCAGCTG 6060

GCACGACAGG TTTCCCGACT GGAAAGCGGG CAGTGAGCGC AACGCAATTA ATGTGAGTTA 6120

GCGCGAATTG ATCTGG 6136

(2) INFORMATION FOR SEQ ID NO:27:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 14"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AGGTCTACTA GTCCCGGCTG CCGCGTCGAC 30

(2) INFORMATION FOR SEQ ID NO:28:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 15"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CCGATTAAAG CTTATTAGCT AGCACGGAAT TCCGTGGGGC TGGTCGTCGG CAC 53

(2) INFORMATION FOR SEQ ID NO:29:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 16"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCATGAGCCA TGGCTAGCGC AAATCTTAAT GGGACGCTGA TG 42

(2) INFORMATION FOR SEQ ID NO:30:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 17"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATGACTAAGC TTACTTACTT AGTGATGGTG ATGGTGATGA CTAGTTCTTT GAACATAAAT TGAAACCGA
69

(2) INFORMATION FOR SEQ ID NO: 31:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1959 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: cDNA
(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..1959
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

ATG GAT CCT AGG ACC ACG CCC GCA CCC GGC CAC CCG GCC CGC GGC GCC 48
Met Asp Pro Arg Thr Thr Pro Ala Pro Gly His Pro Ala Arg Gly Ala
1 5 10 15

CGC ACC GCT CTG CGC ACG ACG CTC GCC GCC GCG GCG GCG ACG CTC GTC 96
Arg Thr Ala Leu Arg Thr Thr Leu Ala Ala Ala Ala Ala Thr Leu Val
20 25 30

GTC GGC GCC ACG GTC GTG CTG CCC GCC CAG GCC GCT AGT CCC GGC TGC 144
Val Gly Ala Thr Val Val Leu Pro Ala Gln Ala Ala Ser Pro Gly Cys
35 40 45

CGC GTC GAC TAC GCC GTC ACC AAC CAG TGG CCC GGC GGC TTC GGC GCC 192
Arg Val Asp Tyr Ala Val Thr Asn Gln Trp Pro Gly Gly Phe Gly Ala

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     50                  55                  60

AAC GTC ACG ATC ACC AAC CTC GGC GAC CCC GTC TCG TCG TGG AAG CTC        240
Asn Val Thr Ile Thr Asn Leu Gly Asp Pro Val Ser Ser Trp Lys Leu
 65                  70                  75                  80

GAC TGG ACC TAC ACC GCA GGC CAG CGG ATC CAG CAG CTG TGG AAC GGC        288
Asp Trp Thr Tyr Thr Ala Gly Gln Arg Ile Gln Gln Leu Trp Asn Gly
                 85                  90                  95

ACC GCG TCG ACC AAC GGC GGC CAG GTC TCC GTC ACC AGC CTG CCC TGG        336
Thr Ala Ser Thr Asn Gly Gly Gln Val Ser Val Thr Ser Leu Pro Trp
            100                 105                 110

AAC GGC AGC ATC CCG ACC GGC GGC ACG GCG TCG TTC GGG TTC AAC GGC        384
Asn Gly Ser Ile Pro Thr Gly Gly Thr Ala Ser Phe Gly Phe Asn Gly
        115                 120                 125

TCG TGG GCC GGG TCC AAC CCG ACG CCG GCG TCG TTC TCG CTC AAC GGC        432
Ser Trp Ala Gly Ser Asn Pro Thr Pro Ala Ser Phe Ser Leu Asn Gly
    130                 135                 140

ACC ACC TGC ACG GGC ACC GTG CCG ACG ACC AGC CCC ACG GAA TTC CGT        480
Thr Thr Cys Thr Gly Thr Val Pro Thr Thr Ser Pro Thr Glu Phe Arg
145                 150                 155                 160

GCT AGC GCA AAT CTT AAT GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC        528
Ala Ser Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr
                165                 170                 175

ATG CCC AAT GAC GGC CAA CAT TGG AAG CGC TTG CAA AAC GAC TCG GCA        576
Met Pro Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala
            180                 185                 190

TAT TTG GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT        624
Tyr Leu Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr
        195                 200                 205

AAG GGA ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT        672
Lys Gly Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    210                 215                 220

GAT TTA GGG GAG TTT CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC        720
Asp Leu Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
225                 230                 235                 240

ACA AAA GGA GAG CTG CAA TCT GCG ATC AAA AGT CTT CAT TCC CGC GAC        768
Thr Lys Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp
                245                 250                 255

ATT AAC GTT TAC GGG GAT GTG GTC ATC AAC CAC AAA GGC GGC GCT GAT        816
Ile Asn Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp
            260                 265                 270

GCG ACC GAA GAT GTA ACC GCG GTT GAA GTC GAT CCC GCT GAC CGC AAC        864
Ala Thr Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn
        275                 280                 285

CGC GTA ATT TCA GGA GAA CAC TTA ATT AAA GCC TGG ACA CAT TTT CAT        912
Arg Val Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His
    290                 295                 300

TTT CCG GGG CGC GGC AGC ACA TAC AGC GAT TTT AAA TGG CAT TGG TAC        960
Phe Pro Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr
305                 310                 315                 320

CAT TTT GAC GGA ACC GAT TGG GAC GAG TCC CGA AAG CTG AAC CGC ATC       1008
His Phe Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile
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                  325                 330                 335

TAT AAG TTT CAA GGA AAG GCT TGG GAT TGG GAA GTT TCC AAT GAA AAC      1056
Tyr Lys Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn
            340                 345                 350

GGC AAC TAT GAT TAT TTG ATG TAT GCC GAC ATC GAT TAT GAT CAT CCT      1104
Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro
        355                 360                 365

GAT GTC GCA GCA GAA ATT AAG AGA TGG GGC ACT TGG TAT GCC AAT GAA      1152
Asp Val Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu
    370                 375                 380

CTG CAA TTG GAC GGT TTC CGT CTT GAT GCT GTC AAA CAC ATT AAA TTT      1200
Leu Gln Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe
385                 390                 395                 400

TCT TTT TTG CGG GAT TGG GTT AAT CAT GTC AGG GAA AAA ACG GGG AAG      1248
Ser Phe Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys
                405                 410                 415

GAA ATG TTT ACG GTA GCT GAA TAT TGG CAG AAT GAC TTG GGC GCG CTG      1296
Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu
            420                 425                 430

GAA AAC TAT TTG AAC AAA ACA AAT TTT AAT CAT TCA GTG TTT GAC GTG      1344
Glu Asn Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val
        435                 440                 445

CCG CTT CAT TAT CAG TTC CAT GCT GCA TCG ACA CAG GGA GGC GGC TAT      1392
Pro Leu His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr
    450                 455                 460

GAT ATG AGG AAA TTG CTG AAC GGT ACG GTC GTT TCC AAG CAT CCG TTG      1440
Asp Met Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu
465                 470                 475                 480

AAA GCG GTT ACA TTT GTC GAT AAC CAT GAT ACA CAG CCG GGG CAA TCG      1488
Lys Ala Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser
                485                 490                 495

CTT GAG TCG ACT GTC CAA ACA TGG TTT AAG CCG CTT GCT TAC GCT TTT      1536
Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe
            500                 505                 510

ATT CTC ACA AGG GAA TCT GGA TAC CCT CAG GTT TTC TAC GGG GAT ATG      1584
Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met
        515                 520                 525

TAC GGG ACG AAA GGA GAC TCC CAG CGC GAA ATT CCT GCC TTG AAA CAC      1632
Tyr Gly Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His
    530                 535                 540

AAA ATT GAA CCG ATC TTA AAA GCG AGA AAA CAG TAT GCG TAC GGA GCA      1680
Lys Ile Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala
545                 550                 555                 560

CAG CAT GAT TAT TTC GAC CAC CAT GAC ATT GTC GGC TGG ACA AGG GAA      1728
Gln His Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu
                565                 570                 575

GGC GAC AGC TCG GTT GCA AAT TCA GGT TTG GCG GCA TTA ATA ACA GAC      1776
Gly Asp Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp
            580                 585                 590

GGA CCC GGT GGG GCA AAG CGA ATG TAT GTC GGC CGG CAA AAC GCC GGT      1824
Gly Pro Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly
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        595                 600                 605

GAG ACA TGG CAT GAC ATT ACC GGA AAC CGT TCG GAG CCG GTT GTC ATC      1872
Glu Thr Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile
    610                 615                 620

AAT TCG GAA GGC TGG GGA GAG TTT CAC GTA AAC GGC GGG TCG GTT TCA      1920
Asn Ser Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser
625                 630                 635                 640

ATT TAT GTT CAA AGA ACT AGT CAT CAC CAT CAC CAT CAC
Ile Tyr Val Gln Arg Thr Ser His His His His His His
                645                 650
```

(2) INFORMATION FOR SEQ ID NO: 32:
  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 653 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear
  (ii) MOLECULE TYPE: protein
  (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

```
Met Asp Pro Arg Thr Thr Pro Ala Pro Gly His Pro Ala Arg Gly Ala
 1               5                  10                  15

Arg Thr Ala Leu Arg Thr Thr Leu Ala Ala Ala Ala Ala Thr Leu Val
            20                  25                  30

Val Gly Ala Thr Val Val Leu Pro Ala Gln Ala Ala Ser Pro Gly Cys
        35                  40                  45

Arg Val Asp Tyr Ala Val Thr Asn Gln Trp Pro Gly Gly Phe Gly Ala
    50                  55                  60

Asn Val Thr Ile Thr Asn Leu Gly Asp Pro Val Ser Ser Trp Lys Leu
65                  70                  75                  80

Asp Trp Thr Tyr Thr Ala Gly Gln Arg Ile Gln Gln Leu Trp Asn Gly
                85                  90                  95

Thr Ala Ser Thr Asn Gly Gly Gln Val Ser Val Thr Ser Leu Pro Trp
            100                 105                 110

Asn Gly Ser Ile Pro Thr Gly Gly Thr Ala Ser Phe Gly Phe Asn Gly
        115                 120                 125

Ser Trp Ala Gly Ser Asn Pro Thr Pro Ala Ser Phe Ser Leu Asn Gly
    130                 135                 140

Thr Thr Cys Thr Gly Thr Val Pro Thr Thr Ser Pro Thr Glu Phe Arg
145                 150                 155                 160

Ala Ser Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr
                165                 170                 175

Met Pro Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala
            180                 185                 190

Tyr Leu Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr
        195                 200                 205

Lys Gly Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr
    210                 215                 220

Asp Leu Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly
225                 230                 235                 240
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Thr Lys Gly Glu Leu Gln Ser Ala Ile Lys S r Leu His Ser Arg Asp
245 250 255

Ile Asn Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp
260 265 270

Ala Thr Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn
275 280 285

Arg Val Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His
290 295 300

Phe Pro Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr
305 310 315 320

His Phe Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile
325 330 335

Tyr Lys Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn
340 345 350

Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro
355 360 365

Asp Val Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu
370 375 380

Leu Gln Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe
385 390 395 400

Ser Phe Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys
405 410 415

Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu
420 425 430

Glu Asn Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val
435 440 445

Pro Leu His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr
450 455 460

Asp Met Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu
465 470 475 480

Lys Ala Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser
485 490 495

Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe
500 505 510

Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met
515 520 525

Tyr Gly Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His
530 535 540

Lys Ile Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala
545 550 555 560

Gln His Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu
565 570 575

Gly Asp Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp
580 585 590

Gly Pro Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly
595 600 605

Glu Thr Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile
610 615 620

Asn Ser Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser
625 630 635 640

Ile Tyr Val Gln Arg Thr Ser His His His His His His
645 650

(2) INFORMATION FOR SEQ ID NO:33:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 18"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CATATGGCTA GCGAATTCGC AAATCTTAAT GGGACGCTG 29

(2) INFORMATION FOR SEQ ID NO:34:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 19"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AAGCTTACTA GTAGGCCTTC TTTGAACATA AATTGAAA 28

(2) INFORMATION FOR SEQ ID NO:35:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 20"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCATGGGCTA GCCCTGAATT CAGGCCTCCA ACCCCCACTA GTCCGAGCGC TCCCAGCGGC
TGCACTGCTG 70

(2) INFORMATION FOR SEQ ID NO:36:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid
(ix) FEATURE:
(A) NAME/KEY: misc-feature:
(B) OTHER INFORMATION: /desc = "Primer 21"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

AGCCTAAGCT TACAGGCACT GATGGTACCA GT 32

2) INFORMATION FOR SEQ ID NO:37:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: protein
(ix) FEATURE:
(a) NAME/KEY: misc-feature
(d) OTHER INFORMATION: /desc = "Linker"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

Arg Pro Pro Thr Pro Thr Ser Pro Ser Ala Pro Ser
1 5 10

CLAIMS

1. A method for liquefying starch, wherein a starch substrate is treated in aqueous medium with a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of an α-amylase linked to an amino acid sequence comprising a carbohydrate-binding domain (CBD).

2. The method for liquefying starch according to claim 1, further comprising a debranching enzyme.

3. The method according to claim 2, wherein the debranching enzyme is a modified debranching enzyme (enzyme hybrid) linked to an amino acid sequence comprising a carbohydrate-binding domain.

4. A method for saccharifying starch which has been subjected to a liquefaction process, wherein the reaction mixture after liquefaction is treated with a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of a debranching enzyme linked to an amino acid sequence comprising a carbohydrate-binding domain (CBD).

5. The method according to claims 2, 3 or 4 wherein said debranching enzyme is an isoamylase or a pullulanase.

6. A method for saccharifying starch which has been subjected to a liquefaction process, wherein the reaction mixture after liquefaction is treated with a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of a glucoamylase linked to an amino acid sequence comprising a carbohydrate-binding domain (CBD).

7. A method according to any one of the preceding claims, wherein said CBD is a CBD deriving from a cellulase, a xylanase, a mannanase, an arabinofuranosidase, an acetylesterase, a chitinase, a glucoamylase or a CGTase.

8. The use of a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of an α-amylase linked to an amino acid sequence comprising a carbohydrate-binding domain (CBD) in a process for liquefying starch.

9. The use of a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of a debranching enzyme linked to an amino acid sequence comprising a carbohydrate-binding domain (CBD) in a process for saccharifying starch which has been subjected to a liquefaction process.

10. The use of a modified enzyme (enzyme hybrid) which comprises an amino acid sequence of a glucoamylase linked to an amino acid sequence comprising a carbohydrate-binding domain (CBD) in a process for saccharifying starch which has been subjected to a liquefaction process.

11. An isolated DNA sequence encoding a hybrid enzyme with amylolytic activity comprising:
(a) a DNA sequence encoding an amylolytic activity;
(b) a DNA sequences encoding a CBD; and
(c) a DNA sequence or fragments thereof encoding the linker sequence shown in SEQ ID no. 21.

12. The isolated DNA sequence according to claims 11, wherein the amylolytic activity is an α-amylase activity, in particular a *Bacillus* α-amylase, especially the activity of Termamyl™ or a variant thereof.

13. The isolated DNA sequence according to claims 11 or 12, wherein the CBD is the CBD of *Bacillus agaradherens* NCIMB No. 40482 alkaline cellulase Cel5A.

14. The isolated DNA sequence according to claim 13, encodes the Termamyl™-linker-Cel5A-CBD encoded by plasmid pMB492 shown in SEQ ID No. 19.

15. The isolated DNA sequence according to claims 11 or 12, wherein the CDB is the CBD-dimer of *Clostridium stercorarium* (NCIMB 11754) XynA.

16. A DNA construct construct comprising the DNA sequence of any of claims 11 to 15 operably linked to one or more control sequences capable of directing the expression of the DNA sequence in a suitable expression host.

17. The DNA construct of claim 16, comprising a nucleotide sequence encoding the promoter selected from the group consisting of the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the promoter of the *Bacillus licheniformis* alpha-amylase gene, the promoter of the *Bacillus amyloliquefaciens* BAN amylase gene, the promoter of the *Bacillus subtilis* alkaline protease gene, or the promoter of the *Bacillus pumilus* cellulase or xylosidase gene.

18. A recombinant expression vector comprising the DNA construct of claims 16 or 17, a promoter, and transcriptional and translational stop signals.

19. A host cell comprising the DNA construct of claims 16 or 17.

20. The cell of claim 19, wherein the cell is a *Bacillus* cell from a strain selected from the group consisting of *B. subtilis*, *B. licheniformis*, *B. lentus*, *B. brevis*, *B. stearothermophilus*, *B. alkalophilus*, *B. amyloliquefaciens*, *B. coagulans*, *B. circulans*, *B. lautus*, *B. megatherium*, *B. pumilus*, *B. thuringiensis* or *B. agaradherens*.

21. A method of producing a CBD/ hybrid enzyme, comprised of culturing the cell of claims 19 or 20 under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.

22. An isolated and purified CBD/enzyme hybrid encoded by the DNA sequence of any of claims 11 to 15.

23. The CBD/enzyme hybrid according to claim 22 being the hybrid enzyme shown in SEQ ID No. 20.

INTERNATIONAL SEARCH REPORT

International application No. PCT/DK 97/00448

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/26, C12N 15/56, C07K 19/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Elsevier Science Ltd, Volume 12, 1994, Edward A. Bayer et al, "The cellulosome - a treasuretrove for biotechnology" --	1-23
Y	US 5496934 A (ODED SHOSEYOV ET AL), 5 March 1996 (05.03.96) --	1-23
Y	WO 9429460 A1 (MIDWEST RESEARCH INSTITUTE), 22 December 1994 (22.12.94) --	1-23
Y	WO 9623874 A1 (NOVO NORDISK A/S), 8 August 1996 (08.08.96) --	1-23

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 February 1998	06 -02- 1998
Name and mailing address of the ISA/ Sw dish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No. PCT/DK 97/00448

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Journal of Bacteriology, Volume 177, No 18, Sept 1995, Nathalie Sauvonnet et al, "Extracellular Secretion of Pullulanase Is Unaffected by Minor Sequence Changes but Is Usually Prevented by Adding Reporter Proteins to Its N- or C-Terminal End" page 5241 - page 5243	2,3,5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

07/01/98

International application No.
PCT/DK 97/00448

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		EP 0695311 A	07/02/96
		FI 954888 A	13/12/95
		IL 109323 D	00/00/00
		JP 8509127 T	01/10/96
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		NZ 265537 A	26/05/97
		US 5670623 A	23/09/97
		WO 9424158 A	27/10/94
WO 9429460 A1	22/12/94	NONE	
WO 9623874 A1	08/08/96	AU 4483496 A	21/08/96
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		EP 0808363 A	26/11/97